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SIMON FLEXNER, M.D.

PEYTON ROUS, M.D.

HERBERT S. GASSER, M.D.

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FIGURES IN THE TEXT



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purchased in the open market under the designation technical casein. The inactivation was done with heat according to Goldblatt and Moritz (3). The cottonseed oil was the technical grade and the irradiation was carried out according to Goldblatt and Moritz (4). The salt mixture was No. 185 of McCollum (5). The lemon juice was decitrated by standing over marble for 24 hours. The corn starch was that sold under the trade name Byron brand and the yeast was labeled tested brewer's yeast-Harris, medicinal. The animals were kept in galvanized iron cages in a room with light and ventilation but no direct sunlight. The tissues at autopsy were fixed in Bouin's fluid and paraffin sections of the sexual and accessory sexual organs were stained with hematoxylin (Harris') and eosin. The diet mixture was made weekly and stored in the ice box. There was an excess of food in the cages at all times. The prepuberal animals were 30 days of age and the postpuberal 100 days of age at the start of the experiment.

RESULTS

Animals were killed or died after 85 (1), 86 (3), 99 (2), and 115 (10) days on the diet. The control animals were killed after 115 days and showed no pathological changes. All experimental animals at death showed clinical xerophthalmia.

The general pathological changes in the experimental animals are entirely similar to those described by Goldblatt and Benischek (2) and Wolbach and Howe (1) and this report will be confined to the reproductive system. Sections have been cut from the seminal vesicle, vas deferens, epididymis, and testis. Step sections (every twentieth section) of the entire prostate were prepared. As a control, sections of the lungs, bronchi, submaxillary gland, tongue, and kidneys were cut to establish the usual changes of vitamin A deficiency.

The testes from all animals were small and the tunical covering wrinkled and relatively thickened. On section the tubules were less abundant, smaller than usual in size, and did not separate as easily as the normal. The vasa deferentia and seminal vesicles showed no significant gross alteration. The prostates were slightly smaller than normal and more compact. On section the tissue was finely granular. In local areas there were foci up to 2 mm. in diameter with increased density and loss of granularity. No cysts were observed.

The seminiferous tubules microscopically are small and lined by a single, rather regular layer of Sertoli cells admixed with a few primary spermatogonia. The latter are for the most part resting but a few show mitotic figures, some of which are atypical. The lumina are

filled with a granular or fibrillar acidophilic non-nucleated debris. The limiting membrane of each tubule is not thickened. The fibrous interstitial tissue and blood vessels are not increased in number nor amount. The interstitial cells of Leydig are apparently increased, more so in the prepuberal than in the postpuberal rats. They do not contain pigment globules. In the postpuberal rats there are multi-nucleated cytoplasmic masses in the tubular lumina, while they are absent in the prepuberal. These cells are similar to those described in the human atrophic testes by Seecof (6). In contrast to some other types of testicular atrophy such as in cryptorchidism and in senility in man there is no thickening of the limiting collagenous membrane and the remaining cells arrange themselves in a definite single layer. The two groups differ in that the prepuberal rats show more apparent increase of interstitial cells of Leydig and the postpuberal rats show giant cells within the tubules. This testicular atrophy in rats with a sufficient supply of vitamin E has also been reported by Evans (7).

Even a cursory examination of the pelvic accessory reproductive glands shows that the changes are more striking and extensive in the prepuberal animals. In one there is atrophy of the epithelium but no cellular infiltration and no metaplasia. In the other seven the changes are similar. The prostatic acini in the middle and anterior regions are dilated and filled with a protein-poor fluid free of cells. The epithelium is low cuboidal and the nuclei are abundant, a picture similar to that of castration (Fig. 1). The large number of nuclei indicates that the cells are not low because of flattening from dilation of the acini. There is no evidence of secretory activity and the typical pale area of the cytoplasm of the normal secreting cell is uniformly absent. There is a slight to moderate increase in the thickness of the stromal collar about each acinus and under low power the lobular markings are more distinct than in the normal gland, indicative of atrophy. In focal areas, most commonly in the middle region, rarely in the anterior region, and never in the posterior region, the lumina are filled with a granular acidophilic debris and numerous polymorphonuclear leucocytes with some cellular infiltration into the surrounding stroma. The epithelium of these glands is of two types: first, a tall columnar nonsecretory cell with pseudostratification of long, slender nuclei (Fig. 2), and second, true squamous epithelium with or without

keratinization (Fig. 3). The process may involve only one alveolus of a branching gland as shown in Fig. 3.

The vasa deferentia show atrophy of both the muscular layer and the epithelium but there is no metaplasia. The intraprostatic portion also is free from metaplasia. The glands associated with this latter portion are free from pathological change. The coagulating gland of three prepuberal animals shows extensive cellular infiltration and metaplasia to the squamous type (Fig. 4).

The vesicular epithelium is atrophic and devoid of the characteristic secretion granules (Fig. 5), but the lumen is filled with a dense homogeneous acidophilic mass, similar to that in the normal vesicle. The duct which connects each vesicle with the urethra shows squamous metaplasia with extensive keratinization in the three positive animals. The epididymal epithelium in three prepuberal animals is atrophic and in one there is degeneration of the cytoplasm and extensive karyorrhexis of the epithelial tubules.

The postpuberal animals show similar changes but of much less severe grade. In the prostate, areas of normal prostatic epithelium with clear areas may be found, while in others it is atrophic. Foci of exudative inflammation with metaplasia are present but small in size and not advanced. In one, the ducts of the glands associated with the ductus deferens show metaplasia. In none is there any change in the coagulating gland. In all there is metaplasia of the vesicular ducts and atrophy of the vesicular epithelium.

These anatomical changes in the accessory sexual glands in terms of physiology mean that the secretion of the male sex hormone is reduced by the feeding of a vitamin A deficient diet, markedly if the feeding is initiated before puberty, slightly, if initiated after puberty. The metaplastic and inflammatory changes in the prostate, vesicular ducts, and deferential glands are entirely similar to those recorded in other organs. The atrophy of the testis with complete suppression of spermatogenesis cannot be due to vitamin E deficiency since this was supplied in adequate amount by means of wheat germ oil.

The Prostate in Vitamin A Deficiency in Man

In a detailed morphological study of 678 prostates by the step section method, five cases of focal metaplasia with an acute exudative

inflammation were encountered. In all, there was an associated disease which produced stenosis of the esophagus; in two a carcinoma of the esophagus, in one a carcinoma of the base of the tongue, in one a bronchial carcinoma, and in one an aneurysm of the aorta. Emaciation was extreme in all cases, but no greater than in an additional four cases of esophageal carcinoma and twenty-one cases of gastric carcinoma with pyloric stenosis which did not show the prostatic lesion.

The histological appearance in the prostate is entirely similar (Fig. 6) to that found in the rats. An entire acinus or a part is lined by stratified flattened cells which at times show keratinization. The lumen is filled with a granular acidophilic material with desquamated cells, lymphocytes and polymorphonuclear leucocytes. The stroma about the acinus is edematous and infiltrated with lymphocytes and an occasional polymorphonuclear leucocyte.

Although these patients probably suffered from a complete vitamin deficiency in addition to protein, carbohydrate, fat, mineral, and water deprivation, it seems probable that vitamin A was the factor in the production of the prostatic lesion. In one of eight cases in male Chinese reported by Sweet and K'ang (8), metaplasia in the prostate was found at autopsy.

This type of metaplasia should not be confused with the metaplasia and atypical hyperplasia which is not infrequently observed in the ducts in senile prostates and commonly in small foci of benign enlargement. This latter type has been discussed by a number of investigators, notably Kasman and Gold (9). However Schmidt (10) in 1907 reported squamous metaplasia and abscess formation in a 5 months old child with keratomalacia and in a 53 year old man with an ulcerating carcinoma of the pylorus, in which it would appear that vitamin A was a factor.

SUMMARY

1. Vitamin A deficiency alone in the white rat is associated with atrophy of the testis and accessory sexual glands. This would appear to be indicative of some disturbance in the hypophyseal-gonadally-prostatic hormonal relationships. All of the known vitamins necessary for the rat, except vitamin A, were present in the diet fed the animals studied.

2. Vitamin A deficiency in the rat is associated with foci of inflammation and epithelial metaplasia in the prostatic acini and vesicular ducts entirely similar to that reported in other organs.

3. Focal metaplasia and inflammation is occasionally encountered in the prostate of patients with extreme inanition associated with stenosis of the esophagus. It seems probable that this lesion is due to vitamin A deficiency.

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EXPLANATION OF PLATE 1

All tissues were fixed in Bouin's solution and stained with Harris' hematoxylin and eosin.

FIG. 1. Cuboidal epithelial cells in the acini of the prostate from a vitamin A deficient rat. $\times 206$.

FIG. 2. Irregular tall epithelium with debris in the lumen in vitamin A deficiency. $\times 158$.

FIG. 3. Squamous metaplasia and keratinization of one branch of an acinus in vitamin A deficiency. $\times 162$.

FIG. 4. Squamous metaplasia and cellular infiltration of coagulating gland in vitamin A deficiency. $\times 128$.

FIG. 5. Low nonsecretory epithelium of the vesicle in vitamin A deficiency. $\times 195$.

FIG. 6. Squamous metaplasia and inflammation in an acinus from a case of stenosis of the esophagus from an aneurysm, possibly due to vitamin A deficiency. $\times 131$.



(Moore and Mark: Effect of avitaminosis-A on prostate)



STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

I. THE CAPACITY OF STRAINS OF PNEUMOCOCCUS TYPE III TO GROW AT 41°C. AND THEIR VIRULENCE FOR RABBITS

By JOHN F. ENDERS, PH.D., AND MORRIS F. SHAFFER,* PH.D.

*(From the Department of Bacteriology and Immunology, The Harvard Medical
School, Boston)*

(Received for publication, April 16, 1936)

Although the well known change from smooth to rough types, with the accompanying loss of antigenic elements, presents an explanation for many instances of loss of virulence by bacteria, it fails to account adequately for differences in the degree of virulence observed among various strains of a species all of which possess the characteristics associated with the smooth form. A notable example of this latter type of variation is found among strains of *Pneumococcus* Type III of human origin when injected into rabbits.

Tillett (1) demonstrated that the majority of strains isolated by him from cases of Type III pneumonia were, although virulent for mice, avirulent for rabbits in the sense that upon injection, even in large numbers, they produced a transient bacteremia which in nearly all cases terminated in recovery. He was able to obtain one strain which killed rabbits in moderate dosage on isolation and which, upon passage in these animals, attained an M.L.D. of 0.0001 cc. when introduced intravenously, but was unable to observe any biological property of the rabbit-virulent strain by which it could be distinguished from the avirulent cultures. Similar differences in the virulence of *Pneumococcus* Type III strains have been noted by others (Levy-Bruhl (2), Watson and Cooper (3)) but no entirely satisfactory explanation of this behavior has been presented.

One of the two principal objectives, then, of these experiments and

* Fellow in Medicine of the National Research Council.

those recorded in the papers which follow has been to reveal attributes of the organisms from several smooth strains of *Pneumococcus* Type III which might account for the differences in virulence. Since the studies of previous workers (*e.g.* Singer and Adler (4), Tillett (5, 6, 7)) on the mechanism of the natural immunity of the rabbit against this microbe do not completely elucidate the means whereby it is removed from the blood stream and destroyed in the body, a second aim of our work has been to investigate further the defensive factors of not only the rabbit but also the mouse and (to a limited extent) of man against infection with this type of pneumococcus.

In the present paper it has been shown that marked differences exist in the ability of various strains of *Pneumococcus* III not only to remain viable, but to multiply at 41°C.— a temperature rapidly attained or exceeded and maintained for varying periods of time by the body of rabbits, following infection. The virulence of these strains for this animal has also been determined with the purpose of revealing a possible correlation between this property and the capacity to proliferate at this temperature.

Materials and Methods

Strains Employed.—13 strains of *Pneumococcus* Type III have been studied. Of these, strain SV,¹ originally isolated by Tillett from a case of lobar pneumonia, is highly virulent for both rabbits and mice. Intradermal injection of 0.001 cc. of a 16 hour blood broth culture into a normal rabbit will bring about the death of the animal. Strain CH was also derived from a case of lobar pneumonia and is the stock strain maintained for several years in this laboratory where it is passed each week through a mouse as routine. Intraperitoneal inoculation of 0.5 cc. of 10⁻⁶ dilution of a 16 hour blood broth culture of CH or SV regularly kills mice in about 48 hours. The remaining 11 strains were isolated from human cases of lobar pneumonia, meningitis, and otitis media within a period of 18 months. 2 of these, Nos. 298 and 312, were studied within a week following their isolation. Stock cultures of these strains are grown in normal defibrinated rabbit's blood under a vaseline seal and kept in the ice box. Transfers are carried out at intervals of 4 to 6 weeks.

Strain SV is passed through a rabbit at intervals of 3 to 4 weeks. The remaining strains were not passed through rabbits prior to the beginning of this work, except in the cases of strains IE and CSp which had been inoculated into rabbits once and recovered in pure culture about 1 year previously. All strains were

¹ Sent to us through the courtesy of Dr. K. Goodner of The Rockefeller Institute.

characterized by the possession of capsules, and produced large mucoid colonies on blood agar. They agglutinated to titer in anti-Type III pneumococcus horse serum and showed no tendency to flocculate in 1/10 dilution of an anti-Type VIII pneumococcus horse serum which agglutinated the homologous type organisms in a titer of 1/80.

The 2 strains of R variants derived respectively from the smooth strains CH and SV were obtained after 8 transfers in 10 per cent anti-Type III pneumococcus rabbit serum infusion broth. These fulfilled all the criteria for the rough form. 0.7 cc. of a 15 hour blood broth culture of each failed to kill mice subsequent to intraperitoneal injection.

Method Used in Determining Growth Curves.—16 hour blood broth cultures of the various strains were diluted in sterile infusion broth. Freshly drawn defibrinated rabbit blood was inoculated with 1 per cent of its volume of a 1/100 dilution of the culture; 2 to 3 cc. of rabbit blood were usually employed. In each case duplicate cultures were made: one was kept at 37°C. in the incubator and the other placed in a water bath at 41°C. \pm 0.1°. At intervals 0.1 cc. samples were removed and dilutions prepared in broth. Measured portions of these were placed in Petri dishes containing 0.5 cc. of defibrinated horse blood and melted infusion agar added. After 48 hours incubation, the colonies were counted. Defibrinated rabbit blood was used as the medium since it would most closely approximate the nutritive environment in the host.

Method of Determining the Virulence for Rabbits.—0.1 cc. of blood broth culture was inoculated into 10 cc. of 0.1 per cent dextrose 0.5 per cent rabbit serum infusion broth. After 7½ hours incubation various amounts of this culture were injected intravenously into normal rabbits. When quantities larger than 1 cc. were tested, the culture was centrifugalized at 2800 R.P.M. for ½ hour and the deposit taken up in 1 cc. of sterile broth which was injected. The number of viable bacteria per 1 cc. of inoculum was determined in each case by the plating method. The temperatures of the animals were recorded each morning and evening.

Variation in the Capacity of 13 Strains of Pneumococcus Type III to Grow at 41°C.

In Table I are presented data which represent the numbers (expressed as the logarithms) of viable organisms per cubic centimeter of defibrinated rabbit blood (incubation at 41°C.), at various intervals following inoculation with each of 13 smooth strains of Pneumococcus Type III and the R variants derived from two of these smooth forms. Figures are also included which denote the numbers of viable organisms present after 24 hours incubation in similar cultures maintained at 37°C. Counts at the latter temperature for earlier intervals were obtained, but are not recorded except in the case of strain SV (whose

TABLE I

Growth of Pneumococcus Type III Strains in Defibrinated Rabbit Blood at 41°C.

Strain	Period of incubation						
	0 hrs.	2½ hrs.	5 hrs.	7½ hrs.	9 hrs.	12 hrs.	24 hrs.
SV (Tillett)	4.21	5.39	7.10	7.85	8.73	n.d.	7.05
	4.49*	5.47*	7.10*	7.96*	8.26*	n.d.	9.38*
CSp	4.60	4.39	6.14	8.03	n.d.	n.d.	7.98
B	4.48	4.40	6.07	7.18	n.d.	n.d.	8.35 9.46*
IE	5.02	4.17	4.08	4.08	n.d.	4.38	5.55
Tirrell	5.97	4.50	4.30	4.39	n.d.	4.91	5.33
CH	3.80	5.11	6.35	6.04	5.28	n.d.	0.00 9.78*
669	4.19	2.48	<2.00	n.d.	n.d.	n.d.	0.00 9.82*
681	4.05	3.54	4.11	3.85	<3.00	n.d.	0.00 9.38*
A305	4.43	3.84	3.86	3.75	n.d.	n.d.	0.00 9.47*
3843	4.20	3.60	3.04	<2.00	n.d.	n.d.	0.00 9.48*
3838	4.39	4.20	4.11	3.69	n.d.	n.d.	0.00 8.96*
298	4.04	2.00	2.00	<2.00	n.d.	n.d.	0.00 9.12*
312	4.40	3.03	2.31	<2.00	n.d.	n.d.	0.00 9.35*
SV (rough form)	4.46	4.34	4.35	5.09	5.79	n.d.	6.57
CH (rough form)	5.48	4.15	3.15	2.30	2.00	n.d.	0.00

Results given as \log_{10} of number of viable pneumococci per cc. of blood.

n.d. = not done.

*Indicates values for duplicate cultures at 37°C.

37°C. values are given as an example) since they showed that the growth rates of all were quite comparable to that of strain SV at this temperature.

From these results it is apparent that individual strains of this type of pneumococcus exhibit marked diversity in their capacity to multiply

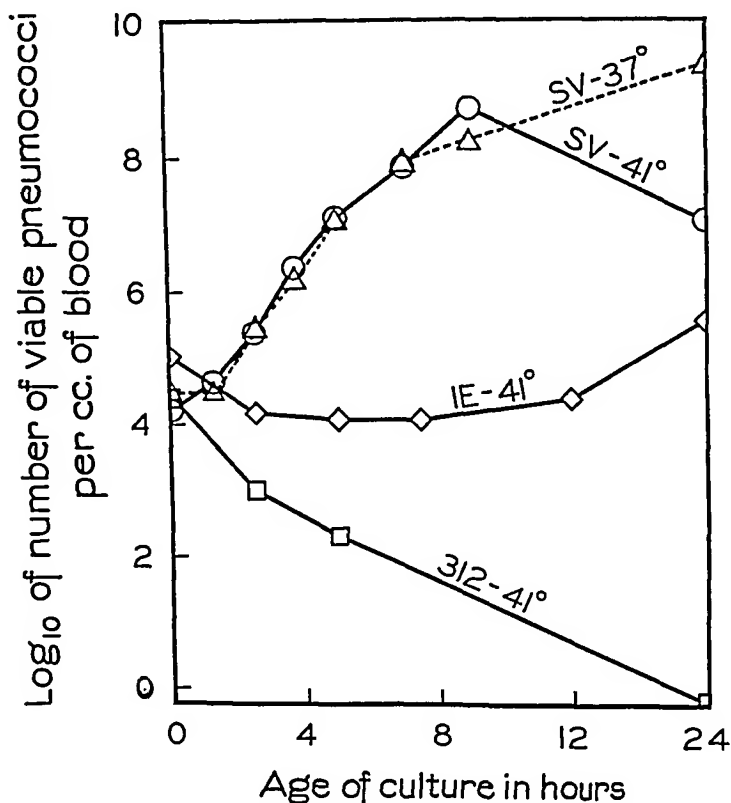


FIG. 1. Growth curves at 41°C. of *Pneumococcus* Type III strains typical of each of the three groups, as well as the SV growth curve at 37°C. ○—○ strain SV (group 1); ◇—◇ strain IE (group 2); □—□ strain 312 (group 3); △-----△ strain SV (37°).

at 41°C. On the basis of such differences it is possible to divide the strains which have been examined into three groups:

1. Those which grow at approximately the same rate and attain almost the same maximal numbers at 41°C. as at 37°C.

2. Those which increase more slowly at 41°C. than at 37°C. or which remain practically stationary at the higher temperature.

3. Those which show little or no evidence of increase at 41°C. and which after a variable interval at this temperature decrease in numbers. This diminution in most instances may progress until no viable organisms remain.

In Text-fig. 1 are presented in the form of growth curves the data for 3 strains each representative of one of these groups. For purposes of comparison the curve for strain SV at 37°C. is included, since this exemplifies the rate of multiplication of all the other strains at this temperature.

It is clear from the figures included in Table I that of the 13 strains studied, 3 clearly fall into the first group, 2 in the second, and 7 in the third. Strain CH, although difficult to classify has been more or less arbitrarily assigned to group 2 since it grew at 41°C. during the early hours following inoculation, but soon the numbers declined and the culture was found to be sterile at 24 hours.

That the growth property at 41°C. of a given strain depends upon stable metabolic processes is indicated by the fact that this remains qualitatively uninfluenced by the S→R change. Thus, it will be seen that the R form derived from strain SV retains the ability to grow at 41°C., whereas that from strain CH, although not exactly reproducing the growth curve obtained with the S form, nevertheless, like the latter, is inhibited at this temperature.

Virulence of the Strains for Rabbits

Our observations on the inability of the majority of our strains to grow well at 41°C. suggested a possible relationship between this behavior and the avirulence for the rabbit of most strains of Pneumococcus Type III reported by other workers, since the temperature of this animal has been found to rise to 41°C. or higher 3 to 5 hours after intravenous infection. Fluctuations occur thereafter but body temperature of 40.3–41.3°C. may be maintained for considerable periods of time. Conversely it seemed probable that the strains which demonstrated their capacity to survive and multiply at this temperature

might prove virulent, particularly since strain SV which was known to be highly virulent for rabbits had been found to grow well at 41°C. Accordingly, a series of rabbits were injected intravenously with the organisms from 5 cc. of a young ($7\frac{1}{2}$ hour) culture at 37°C. of each of 11 strains, the virulence of which was not definitely known. Strains SV and CH were not tested at this time since their relative virulence had been determined in previous experiments.

This preliminary test showed that among the 11 strains, 3 only (IE, CSp, B), were capable of killing rabbits following the injection of 5 cc. of culture. Additional animals were then inoculated with smaller quantities of cultures of these 3 strains. The rabbits injected with 0.1 cc. of the cultures of strains IE and CSp succumbed, while strain B proved to be definitely less virulent since this quantity failed to kill either animal following generalized invasion of the body. Again, of two rabbits each receiving 1 cc. of B culture, only one died and this animal was inoculated with a culture obtained from the heart's blood of the rabbit which had succumbed to 5 cc. These findings, with additional experimental details, are recorded in Table II. It will be seen that all the 7 strains which failed to withstand a temperature of 41°C. (group 3) were not fatal to rabbits in the amounts of culture injected. In contrast both strains (B and CSp) in group 1 were able to bring about death of the animals, although the degree of virulence is obviously not identical. In group 2, of 3 strains only strain IE possessed to a marked degree the property of invading the blood stream and overwhelming the animal. The Tirrell strain possessed little or no virulence, although its growth curve was quite similar to that of strain IE. Certain characteristics exhibited by strain CH which will be described in subsequent communications probably account for its lack of virulence and that of the Tirrell strain. It should be emphasized in respect to the quantities of culture which in these experiments have led to the death of rabbits that although apparently large, they nevertheless indicate a considerable degree of virulence since it has been found by practically all workers (*cf.* Tillett) that the virulence of *Pneumococcus* Type III for rabbits, without repeated passage through these animals, in no instance approaches that of organisms such as *Pneumococcus* Type I.

TABLE II
Virulence for Rabbits of Various Strains of Pneumococcus Type III

Rabbit No.	Weight gm.	Strain Pneumococcus III	Group on basis of growth at 41°C.	No. of organisms in volume of culture injected	Result, death or survival of rabbit	Remarks
1-9	1500	SV	1	0.1 cc. culture	D < 48 hrs.	This dose does not represent the M.L.D. for this strain
7-0	2010	CSp	1	$2.4 \times 10^8 \approx 1.0$ cc.	D < 40 hrs.	2 other animals inoculated. 1.0 cc. and 0.1
4-69	1050	CSp	1	$2.4 \times 10^7 \approx 0.1$ cc.	D < 47 hrs.	cc. of culture CSp passed once through a
2-5	1450	CSp	1	$3.8 \times 10^8 \approx 0.01$ cc.	S	rabbit; both died
8-9	1480	B	1	5 cc. culture	D 96 hrs.	
6-5	2020	B	1	$2.6 \times 10^8 \approx 1.0$ cc.	S	
2-80	1840	B	1	$2.3 \times 10^8 \approx 1.0$ cc.	D 144 hrs.	Pericarditis—large numbers of organisms in the blood; culture passed once through a rabbit
2-1	1730	B	1	$2.3 \times 10^7 \approx 0.1$ cc.	S	Culture passed once through a rabbit
4-74	1020	B	1	$2.6 \times 10^7 \approx 0.1$ cc.	D 192 hrs.	Infection of right eye. Infection of knee joint with pneumococcus—No organisms in heart's blood
3-69	About 1500	IE	2	$4.5 \times 10^8 \approx 5$ cc.	D < 96 hrs.	
2-3	2000	IE	2	$4.5 \times 10^8 \approx 10$ cc.	D 95 hrs.	
3-77	1650	IE	2	$4.5 \times 10^7 \approx 0.1$ cc.	D < 96 hrs.	

		Tirrell Tirrell		2	8 1.8	X 10 ⁸ ≈ 5 cc. X 10 ⁹ ≈ 5 cc.	S D < 96 hrs.	Culture of heart's blood showed only 4 × 10 ⁴ organisms per cc. Few organisms pre- viously found in blood. Doubt as to death from pneumococcus infection 5 hr. culture used for inoculum
2-4 4-75	1610 2150			2 2				
9-2	2500	CH		2	10 cc. culture			
6-69	1710	669		3	3.5 X 10 ⁹ ≈ 5 cc.		S	
4-61	1860	681		3	9.0 X 10 ⁸ ≈ 5 cc.		S	
1-5	1240	A305		3	5 X 10 ⁹ ≈ 5 cc.		S	
1-7	1450	3843		3	2.5 X 10 ⁹ ≈ 5 cc.		S	
1-4	1660	3838		3	4 X 10 ⁹ ≈ 5 cc.		S	
4-67	1620	298		3	1.8 X 10 ⁹ ≈ 5 cc.		S	
4-76	1750	312		3	7.0 X 10 ⁹ ≈ 5 cc.		S	

DISCUSSION

In assessing the significance of these results, we may assert with considerable confidence that those strains which are unable to increase at 41°C., though endowed with the complete antigenic equipment of the smooth virulent organism contained in the capsule, cannot, under the conditions of temperature existing in the body of the rabbit during infection, multiply sufficiently to bring about the death of the animal. We have investigated too few strains to give the results any statistical validity, but those we have obtained suggest that the majority belong to this group. The fact that most *Pneumococcus* Type III strains are not virulent for rabbits is, therefore, entirely in agreement with our findings.

Conversely, evidence for believing that a correlation exists between the ability to withstand 41°C. and virulence is the fact that only among such "thermo-resistant" strains were found those possessing this property of rabbit virulence. It is clear, however, that an additional factor or factors must be involved in determining whether thermo-resistant strains will produce an infection which terminates fatally, since in this class we have found, according to the criteria arbitrarily selected, avirulent, slightly virulent, and virulent varieties. The results of an analysis of one of these factors will be found in the following papers.

The conception that the high normal body temperature of certain species of animals plays a defensive rôle in immunity to infection by microorganisms has been in the minds of workers since the experiments of Pasteur demonstrated that the chicken, normally refractory to anthrax, became susceptible after the temperature had been lowered by immersion in cold water. Nearly as old is the hypothesis that fever itself is an immunological response. Various methods have been employed in attempts to demonstrate experimentally the validity of these hypotheses. They have consisted in depressing or raising the normal body temperature by physical, chemical, or physiological means, either before or after inoculation of the infecting organism.

Experiments of this kind with pneumococci were early carried out. Thus Walther (8), Loewy and Richter (9), Rolly and Meltzer (10) obtained some evidence that raising the body temperature of rabbits to 41–42°C. increased their resistance to infection. Strouse (11), noting that growth of pneumococci was

inhibited at 40–41°C. *in vitro*, lowered the normally high (41°C.) temperature of pigeons by injection of pyramidon and found that these succumbed to infection with quantities of pneumococci which were tolerated by controls. Wadsworth (12) failed to produce pneumonia in rabbits by cooling the animals in water previous to infection; he later (13) tested 25 strains of pneumococci and found that *in vitro* the growth of all was completely inhibited at 40.5–41.2°C. It is difficult to evaluate and to correlate the details of these experiments with our own work since they were obtained before the classification of pneumococci into types was introduced. More recently Findlay (14) in studying the relationship between increased susceptibility to infection and lack of vitamin B in pigeons found that in birds deprived of this vitamin the body temperature was reduced to 103–104°F. and they then succumbed after inoculation with *Pneumococcus* Type II. Wright (15) in his studies of experimental *Pneumococcus* Type I septicemia in rabbits observed that at 40.5°C. the lag period of the virulent organism grown in rabbit blood was definitely increased, but good growth subsequently occurred. He was unable to correlate this transient inhibitory effect of higher temperatures *in vitro* with the removal of the organisms from the blood stream of rabbits, whether the temperature of the animal was previously increased by administration of vaccines or subsequently rose as a result of infection with pneumococci. Levy-Bruhl (2) who studied 20 strains of *Pneumococcus* Type III found that in 0.2 per cent glucose peptone broth all cultures gave at 42°C. a growth which was nearly as abundant and as rapid as at 37°C. On subcutaneous injection in dosage of 1 cc. into rabbits, however, only 4 strains were able to produce a fatal outcome; the other 16 strains did not kill. Although his results concerning the effect of temperature are not in agreement with ours, the proportion of virulent strains is approximately the same.

Our work suggests that the rise in temperature occurring subsequent to infection may be regarded as a most important factor in the natural resistance of the normal rabbit to those strains of Type III pneumococci fully capable of producing fatal disease in man, but which cannot increase sufficiently to bring about the same under the conditions of temperature encountered in the former.

CONCLUSIONS

1. A correlation appears to exist between the failure of certain strains of *Pneumococcus* Type III to grow at 41°C. and their lack of virulence for rabbits.

2. It is likely that the capacity to grow at 41°C.—an attribute constantly but not exclusively associated with strains of *Pneumococcus* Type III virulent for rabbits—is a prerequisite, but not the sole factor, in determining their virulence for these animals.

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EFFECT OF PURIFIED ENZYMES ON VIRUSES AND GRAM-NEGATIVE BACTERIA

By MALCOLM H. MERRILL, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Most of the work on the action of tryptic enzymes on viruses is difficult to interpret since the pancreatic extracts used contained substances other than the enzymes themselves. Pirie (1) has summarized the literature and from her own experiments suggests that the reported inactivation of viruses was due to the fatty acids and lecithins present in the extracts rather than to the enzymes. She was able to inactivate certain viruses by the fatty acids obtained from pancreatic extracts.

Pirie (1) found that crystalline trypsin and chymotrypsin had no effect, or at most a very slight one, on pleuropneumonia, vaccinia, and fowl tumor viruses under the conditions of her experiments. Stanley (2), using crystalline trypsin, found that the reported action of trypsin on tobacco mosaic virus was due to an effect on the host and not on the virus. He further found (3) that this virus was inactivated by pepsin. This last enzyme cannot be used with many animal viruses, since the acidity at which it is active quickly destroys them. Bawden and Pirie (4) have reported the inactivation of the S strain of potato virus, as well as of its power to react with antiserum, by crystalline preparations of trypsin and pepsin.

Since proteolytic enzymes have been obtained in a pure form by Northrop and Kunitz (5), it is now possible to determine more accurately whether viruses are susceptible to their action. The subject is of importance, for if viruses can be inactivated by proteolytic enzymes, it would indicate that they are protein in nature. It would suggest furthermore that they are not living agents, since, according to Northrop (6), all known living cells resist tryptic digestion. It is our purpose to report here experiments made with crystalline trypsin and chymotrypsin on four animal viruses and on a number of Gram-negative bacteria.

Materials and Methods

Drs. Northrop and Kunitz kindly supplied preparations of trypsin and chymotrypsin that had been repeatedly crystallized and then dissolved in sufficient buffer to give an approximate concentration of 1 mg. nitrogen per cc. The pro-enzyme chymotrypsinogen, similarly purified, was used as a control and also after it had been changed to chymotrypsin. The action of these enzymes has been tested on the viruses of equine encephalomyelitis, pseudorabies, swine influenza, and vaccinia.

Since we knew that the first two viruses mentioned are rapidly inactivated at incubator temperature, and since this inactivation can be slowed down by excluding air, all mixtures were kept at refrigerator temperature under vaseline seal. The following procedure was used throughout the work: All solutions were cooled to the temperature of the ice bath before mixtures were made. 5 cc. of the suspension containing virus was mixed with 4.5 cc. of the enzyme solution and 0.5 cc. of 4 per cent sterile neutral cysteine hydrochloride, and melted sterile vaseline was placed on the surface of the mixture. In all experiments except those recorded in Text-fig. 1 and Table II, the final concentration of the enzymes in the mixtures was approximately 0.5 mg. enzyme N per cc. Dilutions were made in salt solution kept at the temperature of an ice bath and inoculations were made immediately after finishing the dilutions.

The stock 4 per cent aqueous solution of cysteine hydrochloride was prepared and adjusted to a pH of 7.3–7.4, passed through a Berkefeld filter, and the filtrate distributed into test tubes and sealed with vaseline. The physiological salt solution used was buffered by adding to each 100 cc., 2 cc. of a stock buffer solution containing 28.81 gm. of NaH_2PO_4 and 125 gm. of Na_2HPO_4 per liter. Tests demonstrated that the cysteine hydrochloride did not inhibit the action of trypsin upon casein.

The activity of the enzyme was always demonstrated by its action on casein and gelatin at the end of the experiment. In all experiments the pH was determined at the beginning and end in order to be sure that inactivation of the viruses was not due to a change in reaction. No marked change occurred.

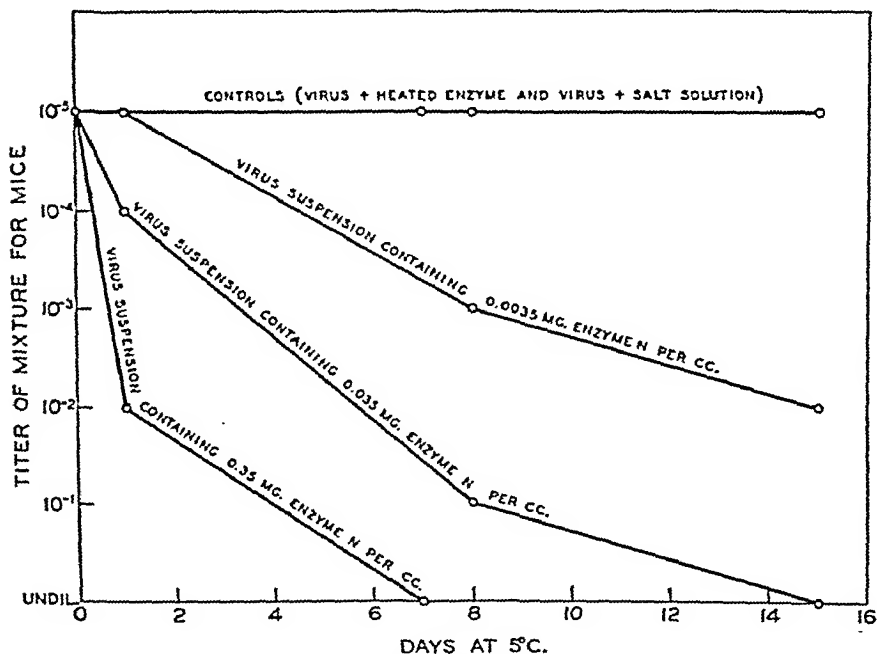
Since enzyme action is markedly slowed at low temperatures, the mixtures were kept for a much longer period of time than would ordinarily be used in digestion experiments. That the enzymes were active under the conditions was repeatedly demonstrated by their action on gelatin and casein.

The criteria for action of the enzyme on the virus were:

1. No immediate inactivation of the virus but inactivation after storage, the degree depending on the length of time stored.
2. Within a given time, the greater the concentration of the enzyme, the more inactivation.
3. No inactivation by chymotrypsinogen nor by boiled trypsin or chymotrypsin.

Effect on Equine Encephalomyelitis Virus

An Eastern strain of equine encephalomyelitis virus isolated in this laboratory was used. Guinea pigs or mice were inoculated intracerebrally, and immediately after death their brains were ground and a 10 per cent suspension made in saline. This suspension was distributed into test tubes, sealed with vaseline, and stored in the refrigerator. The supernatant obtained after centrifugalizing the suspension was mixed with the enzymes and tested by intracerebral injection into guinea pigs as given in the tables.



TEXT-FIG. 1

In Text-fig. 1 are the results of tests made on uniform quantities of virus to which were added decreasing amounts of a mixture of trypsin and chymotrypsin. It will be seen that, judged by the criteria given, the virus was destroyed by the mixture of the two enzymes. Subsequent tests with various preparations of trypsin showed that this enzyme failed to inactivate the virus but that chymotrypsin did so. In Table I are the results of such an experiment. It should be pointed out that the chymotrypsin was prepared from the same lot of chymotrypsinogen used in this experiment by the addition of a small amount of the same trypsin found to be inactive.

In Table II are results which show that the degree of inactivation by chymotrypsin is dependent upon the amount of the enzyme present.

TABLE I
Effect of Enzymes on Equine Encephalomyelitis Virus

Enzyme added	Time after mixing when tested	Dilution of mixture inoculated 0.03 cc. intracerebrally into mice				
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Chymotrypsinogen	Immediately				++	++
	4 days	++	++	++	++	+0
Chymotrypsin	Immediately				++	++
	4 days	++	+0	00	00	00
Trypsin	Immediately				++	++
	4 days			++	+0	++
Chymotrypsin (heat-inactivated) (control)	Immediately				++	00
	4 days			++	++	++
None (control)	Immediately				++	++
	4 days			++	++	++

Each + indicates a mouse that died, and each 0 a mouse that survived.

TABLE II
Effect of Decreasing Amounts of Chymotrypsin on Equine Encephalomyelitis Virus after 6 Days' Storage

Dilution tested	Amount of enzyme N per cc.				Salt solution plus virus (control)
	0.4 mg.	0.04 mg.	0.004 mg.	0.4 mg. boiled	
10 ⁻¹	*	++	++		
10 ⁻²	00	+0	++		
10 ⁻³	00	00	++	++	++
10 ⁻⁴	00	00	+0	++	++
10 ⁻⁵	00	00	00	++	00

Each + indicates a mouse that died, and each 0 a mouse that survived.

* Died immediately from inoculation.

Effect on Pseudorabies Virus

The brain of a rabbit infected with the Aujeszky strain of pseudorabies was suspended and stored in the same way as the equine enceph-

alomylitis virus. The activity of the mixtures was tested by either subcutaneous or intracerebral inoculation of guinea pigs. In Table III the results of one of the tests are given. They show that both chymotrypsin and trypsin inactivate this virus. This inactivation by both enzymes has been repeatedly obtained after storage of the mixtures, whereas immediately after mixing the virus is found to be active.

Effect on Swine Influenza Virus

A suspension of lungs from mice infected with swine influenza was obtained from Dr. Shope. White mice were anesthetized and their noses were immersed in this suspension for approximately 6 seconds. The animals inoculated in this

TABLE III
Effect of Enzymes on Pseudorabies Virus When Tested after 7 Days' Storage at 5°C.

Dilution of mixture*	Result of intracerebral injection of guinea pigs with virus plus				
	Chymotrypsinogen	Chymotrypsin	Trypsin	Heated chymotrypsin (control)	Salt solution (control)
1:5					
1:25		0	0		
1:125		0	0		
1:250	+	0	0		
1:500	+	0	0	+	+
1:1000	0	0	0	+	+
	+	0	0	0	0
				0	+

0 = survived. + = died.

* A 5 per cent suspension of brain from an infected rabbit was used in this experiment.

way either died or were very sick by the 4th day, when their lungs were removed and a 5 per cent suspension of the pulmonary tissue in salt solution was prepared. This suspension was centrifugalized and to the supernatant were added the various enzymes. After 5, 10, and 28 days' storage in the refrigerator samples were withdrawn, diluted, and two mice inoculated with each dilution, in the same manner used for the preparation of the virus.

The results of the one test made are given in Table IV, and it will be seen that there is no indication of destruction of the virus by the various enzymes used. There is a considerable loss of titer after 28 days' storage, but the loss in the control tubes is the same as in those containing active enzymes.

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		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Chymotrypsinogen	Immediately 4 days	++	++	++	++ ++	++ +0
Chymotrypsin	Immediately 4 days	++	+0	00	++ 00	++ 00
Trypsin	Immediately 4 days			++	++ +0	++ ++
Chymotrypsin (heat-inactivated) (control)	Immediately 4 days			++	++ ++	00 ++
None (control)	Immediately 4 days			++	++ ++	++ ++

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	0.4 mg.	0.04 mg.	0.004 mg.	0.4 mg. boiled	
10 ⁻¹	*	++	++		
10 ⁻²	00	+0	++		
10 ⁻³	00	00	++	++	++
10 ⁻⁴	00	00	+0	++	++
10 ⁻⁵	00	00	00	++	00

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	Chymotrypsinogen	Chymotrypsin	Trypsin	Heated chymotrypsin (control)	Salt solution (control)
1:5		0	0		
1:25		0	0		
1:125	+	0	0		
1:250	+	0	0		
1:500	0	0	0	+	+
1:1000	+	0	0	+	+
		0	0	0	0
				0	+

0 = survived. + = died.

* A 5 per cent suspension of brain from an infected rabbit was used in this experiment.

way either died or were very sick by the 4th day, when their lungs were removed and a 5 per cent suspension of the pulmonary tissue in salt solution was prepared. This suspension was centrifugalized and to the supernatant were added the various enzymes. After 5, 10, and 28 days' storage in the refrigerator samples were withdrawn, diluted, and two mice inoculated with each dilution, in the same manner used for the preparation of the virus.

The results of the one test made are given in Table IV, and it will be seen that there is no indication of destruction of the virus by the various enzymes used. There is a considerable loss of titer after 28 days' storage, but the loss in the control tubes is the same as in those containing active enzymes.

Effect on Vaccinia Virus

In the first two experiments a suspension was used of the testicles of a rabbit inoculated with the New York State Board of Health strain of vaccinia virus. There was no definite evidence of inactivation by either trypsin or chymotrypsin, the mixtures being kept for 3 weeks under the conditions described above.

A test was then made using a washed suspension of elementary bodies obtained from Dr. T. M. Rivers. This suspension was diluted 1:5, and 0.5 cc. of ether was added to each 10 cc. of suspension. Equal amounts of this suspension and of the enzyme preparations were mixed and tested by intracutaneous inoculation into rabbits, as given in Table V. The results show no inactivation by chymotrypsin, slight inactivation by trypsin, and a more pronounced inactivation by a mixture of equal parts of trypsin and chymotrypsin.

TABLE VI
Effect of Trypsin on Gram-Negative Bacilli

Bacterium tested	Bacterial count after 10 days at 5°C.	
	In the presence of trypsin	Control
<i>B. bovissepticus</i>	1.0×10^8	2.5×10^8
<i>B. coli</i> II (non-motile).....	5.9×10^8	2.7×10^8
<i>B. coli</i> III (motile).....	9.9×10^8	9.2×10^8
<i>B. enteritidis</i>	1.6×10^9	1.2×10^9
<i>B. paratyphosus</i> A.....	3.7×10^8	2.2×10^8
<i>B. paratyphosus</i> B.....	4.3×10^8	3.8×10^8
<i>B. pseudotuberculosis</i>	4.8×10^8	2.7×10^8
<i>B. pullorum</i>	1.5×10^9	2.1×10^9
<i>B. suispestifer</i>	2.0×10^8	1.9×10^8
<i>B. typhosus</i>	9.0×10^7	9.0×10^7

Action on Gram-Negative Bacteria

In the older literature (7) it is stated quite definitely that enzymes do not digest living Gram-negative bacteria, but recent papers by Bruner (8) and by Day and Gibbs (9) raise the question of whether proteolytic enzymes do not after all digest the organisms. Because of these recent papers and also because there are no reports on the action of purified enzymes on bacteria, it seemed advisable to restudy this subject.

In Table VI are the results of an experiment on the effect of trypsin on ten different Gram-negative bacteria. The conditions were the

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same as with the viruses, and it will be seen that under these conditions there was no destruction of any of the organisms. Other experiments, which need not be given in detail, were made with chymotrypsin and trypsin, and in mixtures kept up to 30 days there was no evidence of digestion of the organisms. When added to nutrient broth either trypsin or chymotrypsin apparently increased the growth of every organism tested. Killed Gram-negative bacteria were rapidly digested by either enzyme, while Gram-positive organisms, either living or dead, resisted the action of both enzymes.

DISCUSSION

The results given show that viruses differ in their resistance to inactivation by tryptic enzymes. Equine encephalomyelitis virus is inactivated by chymotrypsin and not by trypsin; vaccinia is slowly inactivated by trypsin, but chymotrypsin does not affect its activity; pseudorabies virus is inactivated by both enzymes; and swine influenza virus is inactivated by neither.

Although not recorded in the tables, it was shown in every case in which viruses were inactivated that the inactive mixtures when mixed with fresh virus did not influence the strength of the latter. This is evidence that the digestion products were not responsible for the inactivation. Since the viruses were not in a pure state it is impossible to say that inactivation and digestion are the same, but it is possible to say that some viruses behave like proteins in that they are susceptible to the action of proteolytic enzymes.

The fact that some viruses are not inactivated by the enzymes used does not rule out the possibility that they too may be protein in nature. If we had other enzymes it might be found that these resistant viruses were inactivated by them, or it may be that, like killed Gram-positive bacteria, they resist the action of the trypsin.

The bacteria tested behave like other known living organisms in that they resist digestion with tryptic enzymes. Some of the viruses are inactivated, on the other hand. This can be taken as presumptive evidence that the viruses are non-living. In this connection one may recall the fact that Stanley (10) has isolated a crystalline protein which has the properties of tobacco mosaic virus. The findings suggest the possibility of classifying viruses according

to their resistance to trypsin and other enzymes. Such a classification would be useful when it is determined how these various enzymes differ in their action, for it might throw light on the structure of the viruses.

SUMMARY

Evidence is presented that some viruses behave like proteins in that they are inactivated by proteolytic enzymes, whereas others prove more or less resistant. Ten strains of living Gram-negative bacteria resisted the action of purified trypsin and chymotrypsin, while the killed organisms were rapidly digested. Gram-positive bacteria, on the other hand, were resistant whether living or dead. The findings are discussed.

Drs. Northrop and Kunitz not only supplied the enzyme preparations but were generous in giving advice and criticism.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

X. THE IMMUNOLOGICAL PROPERTIES OF AN ARTIFICIAL ANTIGEN CONTAINING GLUCURONIC ACID

BY WALTHER F. GOEBEL, PH.D.

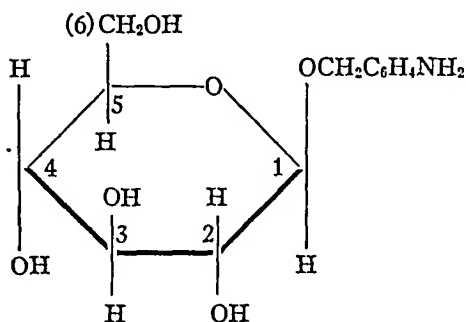
(From the Hospital of The Rockefeller Institute for Medical Research)

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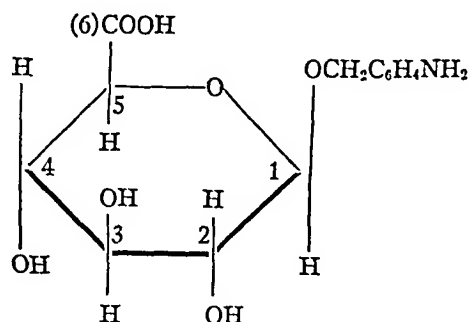
In previous studies it has been shown that differences in the specificity of artificially compounded carbohydrate-protein antigens can be directly correlated with changes in the stereochemical configuration of the carbohydrate radicals (1). Configurational differences alone, however, do not account for all variations in the immunological specificity of carbohydrates. It has been observed that structural changes, such as the presence of an acetyl group in the naturally occurring bacterial polysaccharides, or in the glycoside radical of artificially prepared carbohydrate-protein antigens, are likewise reflected in serological specificity (2).

The uronic acids have been found to be constituents of the specific polysaccharides of *Pneumococcus* (3) and of Friedländer's bacillus (4). The occurrence of glucuronic acid and its isomers in these bacterial carbohydrates has led to the opinion that the highly polar carboxyl group of the uronic acids plays an important rôle in determining the specificity and serological reactivity of the soluble specific substances of encapsulated microorganisms. In support of this view it has been shown that the specific polysaccharide of Type I *Pneumococcus* may be rendered serologically inert merely by esterifying the carboxyl groups with diazomethane (5). The immunological activity of the carbohydrate may be restored, however, when the carboxyl groups are set free by saponifying the methyl ester with dilute alkali. It has been suggested, furthermore, that the immunological crossing exhibited by the specific polysaccharides of Type III and VIII *Pneumococcus* may be attributed to the configurational identity of the aldobionic acid nucleus common to both carbohydrates (6).

In order to study the immunological properties of uronic acids of known constitution, an artificial carbohydrate-protein antigen containing glucuronic acid has been prepared by combining the diazonium derivative of the *p*-aminobenzyl glucuronide with protein. The immunological properties of this antigen have been compared with those of a similar antigen containing the corresponding glycoside of glucose. Since the carbohydrate radicals of these two antigens have an identical stereochemical configuration, any differences in immunological properties must be directly attributable to constitutional changes brought about by differences in the chemical grouping occupying the sixth position in each glycoside molecule. From the accompanying structural formulae of the two *p*-aminobenzyl glycosides, it can be seen that in the glucoside this grouping is a primary alcohol (CH_2OH), whereas the highly polar carboxyl group (COOH) occupies the sixth position in the glucuronide.



p-Aminobenzyl β -glucoside



p-Aminobenzyl β -glucuronide

Chemical Methods

Tetracetyl p-Nitrobenzyl β -Glucoside.—This glucoside was prepared by shaking an ethereal solution of 15 gm. of acetobromoglucose (7) with 1.2 mols of *p*-nitrobenzyl alcohol and 1.2 mols of silver oxide until the ethereal solution no longer showed the presence of free bromo compound. After filtering and concentrating the solution *in vacuo*, the glucoside crystallized on the addition of ethyl alcohol; 5.3 gm. of glucoside were recovered. The glucoside was recrystallized from alcohol. The substance separated as needles melting at $132\text{--}133^\circ$ (uncorrected).

Rotation— $[\alpha]_D^{24} = -40.9^\circ$ in CHCl_3 ($C = 1.0$ per cent).

Analysis— $\text{C}_{13}\text{H}_{13}\text{O}_8(\text{COCH}_3)_4\text{N}$. Calculated. C 52.2, H 5.2.

Found. " 52.4, " 5.3.

p-Nitrobenzyl Glucoside—10 gm. of tetracetyl *p*-nitrobenzyl β -glucoside were suspended in 200 cc. of absolute methyl alcohol at 0° and deacetylated by treating with $1/30$ mol of barium methylate, according to the method of Isbell (8). After

removing the barium by adding the equivalent quantity of $N/1$ sulfuric acid, the glucoside was recovered nearly quantitatively from the mother liquors. The compound crystallized as needles melting at $156-157^\circ$ (uncorrected).

Rotation— $[\alpha]_D^{24} = -47.7^\circ$ in CH_3OH ($C = 0.7$ per cent).

Analysis— $C_{13}H_{11}O_5N$. Calculated. $C\ 49.5, H\ 5.4$.
Found. " $49.8, " 5.3$.

p-Aminobenzyl Glucoside—5 gm. of *p*-nitrobenzyl glucoside were dissolved in 150 cc. of absolute methyl alcohol and reduced catalytically with hydrogen and platinum (9). On concentrating the mother liquors the glucoside crystallized as needles melting at $142-143^\circ$ (uncorrected).

Rotation— $[\alpha]_D^{26} = -61.8^\circ$ in H_2O ($C = 0.8$ per cent). Further recrystallization failed to change the melting point or rotation.

Analysis— $C_{13}H_{13}O_6N$. Calculated. $C\ 54.7, H\ 6.7, N\ 4.9$.
Found. " $54.9, " 7.1, " 4.7$.

p-Aminobenzyl Glucuronide—2.0 gm. of the *p*-nitrobenzyl glycoside of glucuronic acid methyl ester (10) were dissolved in 100 cc. of absolute methyl alcohol and reduced to the amino derivative catalytically with hydrogen and platinum oxide. After removing the platinum by filtration and concentrating the mother liquors, the *p*-aminobenzyl glycoside of glucuronic acid methyl ester separated from the solution as an amorphous snow white flocculent precipitate. The glycoside was dissolved in water and treated with exactly one equivalent of $0.4\ N$ barium hydroxide at 60° , or sufficient base to hydrolyze the methyl ester grouping and to form the barium salt of the *p*-aminobenzyl glucuronide. The latter was isolated by concentrating the solution to small volume *in vacuo*, and precipitating the barium salt with 10 volumes of methyl alcohol. The salt was filtered and dried to constant weight.

Rotation— $[\alpha]_D^{23} = -71.2^\circ$ in H_2O ($C = 1.0$ per cent).

Analysis— $(C_{12}H_{16}O_5NCOO)_2Ba$. Calculated. $Ba\ 18.7, C\ 42.5, H\ 4.4, N\ 3.8$.
Found. " $18.5, " 41.9, " 4.6, " 3.6$.

Immunological Reactions

Methods.—The immunizing antigens were prepared by combining the diazonium derivative of the *p*-aminobenzyl glycoside of glucose and the sodium salt of the corresponding glycoside of glucuronic acid with the globulin of horse serum in alkaline solution. The resulting azoprotein antigens were purified in the usual way, and were finally diluted with isotonic salt solution to a concentration of 0.5 per cent. Rabbits were immunized by the intravenous injection of 1 cc. of these solutions daily for six doses. After a rest period of 1 week, the course of injections was repeated. 8 days after the last injection the rabbits were bled and the sera tested for homologous and heterologous precipitins. The technique of the precipitin and inhibition tests is the same as that described in earlier papers. In the specific inhibition tests, the *p*-aminobenzyl glycoside of glucuronic acid was used in the form of its sodium salt, prepared by adding to a solution of the barium salt the equivalent quantity of solid sodium sulfate. The barium sulfate was removed by centrifugation.

Specific Precipitin and Inhibition Tests.—The sera of rabbits immunized respectively with the azoprotein antigens containing the benzyl glycosides of glucose and glucuronic acid were tested for the presence of homologous and heterologous precipitins. In order to eliminate any interference from common protein antibodies, the *test* antigens were prepared by combining the same glycosides with the proteins of chicken serum in the usual manner. The results of the precipitin tests are given in Table I. From the results given in Table I it can be seen that the two carbohydrate-protein antigens give rise in rabbits to antibodies which are specific and show no serological

TABLE I

Precipitins in Sera of Rabbits Immunized with Glucose and Glucuronic Acid Antigens

Antiserum prepared by immunization with	Test antigen used	Final dilution of test antigen			
		1:2000	1:10,000	1:50,000	1:100,000
Glucose-globulin*	Glucose-chick	++	++++	+++	++
	Glucuronic acid-chick	0	0	0	0
Glucuronic acid-globulin	Glucose-chick	0	0	0	0
	Glucuronic acid-chick	++	+++±	+++	++

* For the sake of brevity the immunizing antigens, prepared by combining the *p*-aminobenzyl glycosides of glucose and glucuronic acid with normal horse serum globulin, are referred to as glucose-globulin, etc. The test antigens, prepared by combining the same glycosides to chicken serum proteins, are referred to as glucose-chick, etc.

crossing. The specificity of these serological reactions is further emphasized by the results of the specific inhibition tests given in Table II, in which it may be seen that the precipitation of the glucose test antigen in homologous antiserum is inhibited only by the glucoside, whereas the specific reaction of the glucuronic acid antigen in homologous antiserum is inhibited only by the glucuronide.

It has previously been pointed out that the carbohydrate radicals of the artificial antigens differ from each other only in the nature of the chemical grouping on the sixth carbon atom of each glycoside. Although the stereochemical configuration of the asymmetric carbon atoms of the two glycosides is in each instance identical, yet this

configurational identity is not reflected in the specificity of the antibodies to which the conjugated protein-glycosides give rise. The distinct and sharply defined specificity of these two antigens appear, therefore, to be directly attributable to differences in polarity of the primary alcohol group in the glucoside molecule, and the carboxyl group in the glucuronide molecule.

Precipitin Reactions of Glucuronic Acid and Glucose Antigens in Antipneumococcus Sera Types II, III, and VIII.—The opinion has frequently been expressed in communications from this laboratory that the uronic acid constituents of the specific polysaccharides of encapsu-

TABLE II

Inhibition of Precipitin Reactions of Glucose and Glucuronic Acid Antigens in Homologous Sera by p-Aminobenzyl Glycosides of Glucose and Glucuronic Acid

Antiserum prepared by immunization with		0.9 per cent salt solution	Inhibiting glycoside		Test antigens (1:5000)		Result
			$\mu/10$ p-amino-benzyl glucoside	$\mu/10$ p-amino-benzyl glucuronide	Glucose-chick	Glucuronic acid-chick	
	cc.	cc.	cc.	cc.	cc.	cc.	
Glucose-globulin	0.2	0.3	0.3	0.3	0.5		+++±
	0.2				0.5		0
	0.2				0.5		+++±
Glucuronic acid-globulin	0.2	0.3	0.3	0.3		0.5	+++±
	0.2					0.5	+++±
	0.2					0.5	0

lated bacteria are important in determining the specificity of the latter, and that the carboxyl groups of the polysaccharide may actually enter into chemical combination with reactive groups in the homologous antibody molecule. The specificity of these serological reactions is believed to be governed by the configurational relationship of the specifically reacting groups in the homologous antibody and carbohydrate molecules.

The important function of the uronic acids of bacterial polysaccharides in determining immunological specificity is strikingly emphasized by the results given in Table III. It may be seen that the glu-

curonic acid-protein antigen reacts in high dilutions with Types II, III, and VIII antipneumococcus horse sera, whereas the corresponding glucose-protein antigen is serologically inert. It has been found in this laboratory that the specific carbohydrates of Types III and VIII (and probably Type II) Pneumococcus contain glucuronic acid as an important constituent of the polysaccharide molecule. The Type I

TABLE III

Precipitin Reactions of Glucose and Glucuronic Acid Antigens in Antipneumococcus Horse Sera Types I, II, III, and VIII

Antipneumococcus horse serum	Test antigen used	Final dilution of test antigen			
		1:10,000	1:50,000	1:200,000	1:1,000,000
Type					
I	Glucose-chick	0	0	0	0
II	" "	0	0	0	0
III	" "	+	+	0	0
VIII	" "	0	0	0	0
I	Glucuronic acid-chick	0	0	0	0
II	" " "	++	++±	+	0
III	" " "	++++	++++	+++±	+
VIII	" " "	++	++±	++	±

TABLE IV

Precipitin Reaction of Glucuronic Acid Test Antigen in Unabsorbed and Absorbed Type III Antipneumococcus Serum

Antipneumococcus horse serum Type III	Final dilution of glucuronic acid test antigen		
	1:2000	1:10,000	1:100,000
Unabsorbed	++	++++	++++
Absorbed with SSS III	0	0	0

pneumococcus specific carbohydrate, on the other hand, apparently contains galacturonic acid. The precipitation of the artificial antigen in Types II, III, and VIII antipneumococcus horse serum may be attributed to the interaction of the glucuronic acid radical of the azo-protein with antibodies elicited by the uronic acid groupings of the bacterial polysaccharides. It may be seen in Table IV that if the

type-specific antibodies in pneumococcus serum Type III are first removed by absorption with the soluble specific substance, the absorbed serum fails to react with the artificial glucuronic acid-protein antigen. It is evident, therefore, that the precipitation of the artificial antigen in antipneumococcus serum Type III (and probably in Types II and VIII as well) represents a reaction between the type-specific polysaccharide antibodies and the uronic acid radical of the glucuronic acid-protein antigen. This fact is emphasized by the

TABLE V

Inhibition of Precipitin Reaction of Glucuronic Acid Antigen in Antipneumococcus Sera Types II, III, and VIII, by p-Aminobenzyl Glycosides of Glucose and Glucuronic Acid

Antipneumococcus horse serum		0.9 per cent NaCl solution	Inhibiting glycoside		Glucuronic acid-chick test antigen (1:5000)	Result
			$\pi/10$ p-aminobenzyl glucoside	$\pi/10$ p-aminobenzyl glucuronide		
Type	cc.	cc.	cc.	cc.	cc.	
II	0.2	0.3	0.3	0.3	0.5	+++
	0.2				0.5	++
	0.2				0.5	0
III	0.2	0.3	0.3	0.3	0.5	++++
	0.2				0.5	++++
	0.2				0.5	0
VIII	0.2	0.3	0.3	0.3	0.5	+++
	0.2				0.5	+++
	0.2				0.5	0

results given in Table V, in which it may be seen that the serological activity of the glucuronic acid-protein antigen in antipneumococcus sera Types II, III, and VIII is completely and specifically inhibited by the addition of the p-aminobenzyl glycoside of glucuronic acid, but not by the corresponding glycoside of glucose.

In contrast to these observations, it has been found (Table VI) that when Type III antipneumococcus serum is absorbed with the glucuronic acid antigen, the antibodies reactive with the homologous capsular polysaccharide are not completely removed. It has been

found, furthermore, that the absorbed serum still reacts with the heterologous Type VIII pneumococcus polysaccharide.

Although the glucuronic acid-protein antigen reacts in antipneumococcus horse sera Types II, III, and VIII, no serological reactions occur in the corresponding antipneumococcus rabbit sera. The sera of rabbits which have been immunized with the glucuronic acid-protein antigen do not agglutinate Types II, III, or VIII pneumococci, nor do they precipitate the corresponding specific capsular polysaccharides.

TABLE VI

Precipitin Reactions of Specific Polysaccharides of Types III and VIII Pneumococcus in Type III Antipneumococcus Serum Absorbed with Glucuronic Acid Antigen

Antipneumococcus serum Type III	Specific polysaccharide used as test antigen	Final dilution of specific polysaccharide				
		1:200,000	1:1,000,000	1:2,000,000	1:4,000,000	1:6,000,000
	Type					
Unabsorbed	III	++++	++±	++	±±	+
Absorbed with glucuronic acid-chick	III	++++	++±	++	±±	+
Unabsorbed	VIII	+++	++	+	±	0
Absorbed with glucuronic acid-chick	VIII	+++	++	+	±	0

DISCUSSION

The important rôle played by acid groups in determining the specificity of certain azoproteins has been emphasized and extensively investigated by Landsteiner and his coworkers (11). In the case of azoproteins containing substituted aromatic nuclei, it has been especially well demonstrated that the nature of the acid groups and their relative position in the benzene nucleus are important factors in determining serological specificity. It is not surprising, therefore, to find that artificial antigens containing the azobenzyl glycosides of glucose and glucuronic acid show separate and distinct specificities. Each glycoside contains four asymmetric carbon atoms. The *p*-aminobenzyl glycosides of glucose and glucuronic acid have an identical stereochemical configuration, however, and differ from one another

only in the nature of the chemical grouping occupying the sixth position. In the case of the glucoside this grouping is a primary alcohol (CH_2OH), whereas in the glucuronide a highly polar carboxyl (COOH) group occupies this position. This difference in molecular structure suffices to confer distinct and specific serological characteristics upon antigens containing these two carbohydrate radicals. It is remarkable that the antisera to these two antigens show no serological crossing despite the identity in configuration of the asymmetric carbon atoms of each carbohydrate. The immunological specificity exhibited by both antigens appears referable, therefore, to a distinct individuality conferred upon each by changes in the chemical grouping occupying the sixth position in each hexoside radical.

The important rôle played by acid groups in determining the serological specificity of certain bacterial polysaccharides is likewise emphasized by the precipitin reaction of the glucuronic acid antigen in antipneumococcus horse sera Types II, III, and VIII. In this respect the artificial glucuronic acid-protein antigen bears a striking immunological relationship to the specific bacterial polysaccharides, though the chemical relationship resides solely in the common uronic acid constituent. In preliminary reports of this investigation (12), it was pointed out that the serological activity of the artificial glucuronic acid-protein antigen in antipneumococcus sera might be attributed to the interaction of the antigen with antibodies related to the uronic acid constituents of the bacterial polysaccharides. Further evidence in support of this point of view has since been presented by Marrack (13) and his associates, who have found that an artificial azoprotein antigen containing the naturally occurring glycoside of glucuronic acid; euxanthic acid, reacts in high dilutions in Type II antipneumococcus serum.

That the serological activity of the glucuronic acid antigen in antipneumococcus sera Types II, III, and VIII represents a reaction between the artificial antigen and the specific carbohydrate antibodies in these sera is clearly substantiated by the results of the specific absorption and inhibition tests. Despite the intimate serological relationship which the glucuronic acid antigen bears to the capsular polysaccharides of Types II, III, and VIII pneumococcus, yet it is not possible to remove from antipneumococcus serum Type III the carbohydrate

antibodies by absorption with the artificial antigen. This lack of complete reciprocal absorption is not fully understood, but it is possible that quantitative precipitin studies may throw light on this perplexing question.

SUMMARY

1. Artificial carbohydrate-protein antigens containing the azobenzylglycosides of glucose and glucuronic acid give rise in rabbits to antibodies which are distinct and immunologically specific.

2. The artificial antigen containing glucuronic acid reacts in high dilutions in antipneumococcus horse sera Types II, III, and VIII. The chemical basis for this serological activity is discussed.

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AN AIR-DRIVEN ULTRACENTRIFUGE FOR MOLECULAR SEDIMENTATION

By J. BISCOE, E. G. PICKELS, PH.D., AND RALPH W. G. WYCKOFF, PH.D.
(From the Laboratories of The Rockefeller Institute for Medical Research, and the
Laboratories of the International Health Division of the Rockefeller
Foundation, New York)

PLATES 2 TO 4

(Received for publication, April 14, 1936)

Centrifuging at very high speeds is one of the most fruitful methods for studying large molecules in solution. Apparatus for carrying out such studies has been developed by Svedberg¹ over the last 12 years and has been used by him and his students in the investigation of a large number of macromolecular systems. In this equipment high rotational speeds are obtained by driving a large steel rotor with a turbine using oil under several atmospheres pressure. Such a machine is so elaborate and costly both to build and to maintain that as yet no duplicates of it have been put in operation.

The development of usable air-driven turbines for high rotational speeds² has opened up the possibility of making comparatively inexpensive ultracentrifuges. One such air-driven machine for molecular sedimentation³ has already been described; it has the disadvantage with respect to Svedberg's that it uses much smaller rotors and is accordingly incapable of yielding results of equal accuracy. The apparatus described in the present paper is one that we have been developing to provide data needed in studying viruses and crystalline proteins. It has been built in our own shop and the materials necessary for its construction, including the lenses, have cost less than one thousand dollars. In designing this ultracentrifuge we have sought

¹ See Svedberg, T., *Naturwissenschaften*, 1934, 22, 225 for bibliography.

² See Beams, J. W., and Pickels, E. G., *Rev. Scient. Instruments*, 1935, 6, 299 for bibliography.

³ McBain, J. W., and O'Sullivan, C. M., *J. Am. Chem. Soc.*, 1935, 57, 2631.

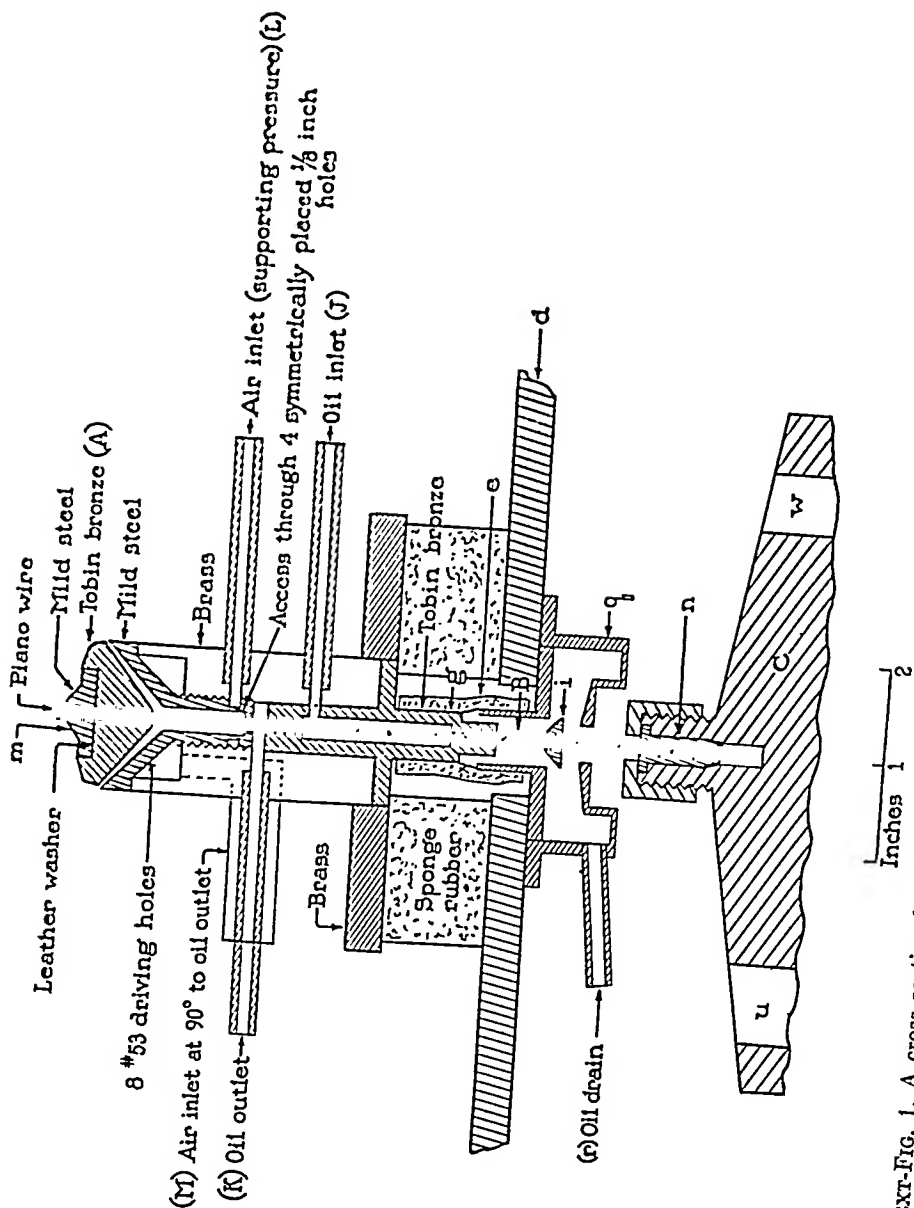
to utilize, to the greatest possible extent, the experience embodied in the publications of Svedberg. We have therefore copied his optical system directly and have employed large rotors capable of giving the 6.5 cm. distance between cell center and rotation axis which he has adopted for his most accurate measurements.

In all forms of the ultracentrifuge for the sedimentation of molecules in solution, a small volume of this solution is enclosed in a cell that can be rotated at high speeds. If the dissolved substance has a density different from that of the solvent, there will be a gradual separation of the two kinds of molecules due to the intense "gravitational" field produced by the rotation. The extent of this separation can be measured by photographing through the moving cell. Two methods for carrying out this photography have been devised, one based on changes in absorption, the other on variations in refractive index. We are using the former in which the chosen light is such that it is transmitted by the solvent but absorbed by the solute. By measuring in this way the rate or the extent of the separation of solvent and solute data are obtained that give an indication of the weight and shape of the dissolved molecules. From this outline it can be seen that the essentials of an ultracentrifuge consist of (1) a rotor containing the cell and its solution so mounted that it can turn at high speeds, (2) a driving mechanism for the rotor and (3) a camera and suitable light source for obtaining pictures through the rotating solution.

General Description

The general arrangement of these essential parts in our apparatus, similarly lettered in the drawing and the pictures, is shown in Text-fig. 1 and Figs. 1-4. The driving mechanism, patterned after that of Beams and Pickels,² is a small bronze air turbine (*A*). Attached to this turbine is a steel shaft (*B*) that, passing through an oil-sealed bearing (*g*), supports the large duralumin rotor (*C*) turning in the vacuum chamber (*D*). In obtaining photographs of the rotating solution, suitably filtered light from a mercury vapor arc (*E*) passes into the vacuum chamber through a bottom quartz window, then intermittently through the solution cell in the turning rotor, out through another quartz window (*a*) and along the camera tube (*F*) to be registered on a plate in the holder (*G*).

A cross section through the driving mechanism is shown in Text-fig. 1. Details of construction not shown in this figure, such as the nature of the flutings to be milled in the turbine and the positions of the air ports in the conical driver, are to be found in the paper by Beams and Pickels. The necessary air-tight but



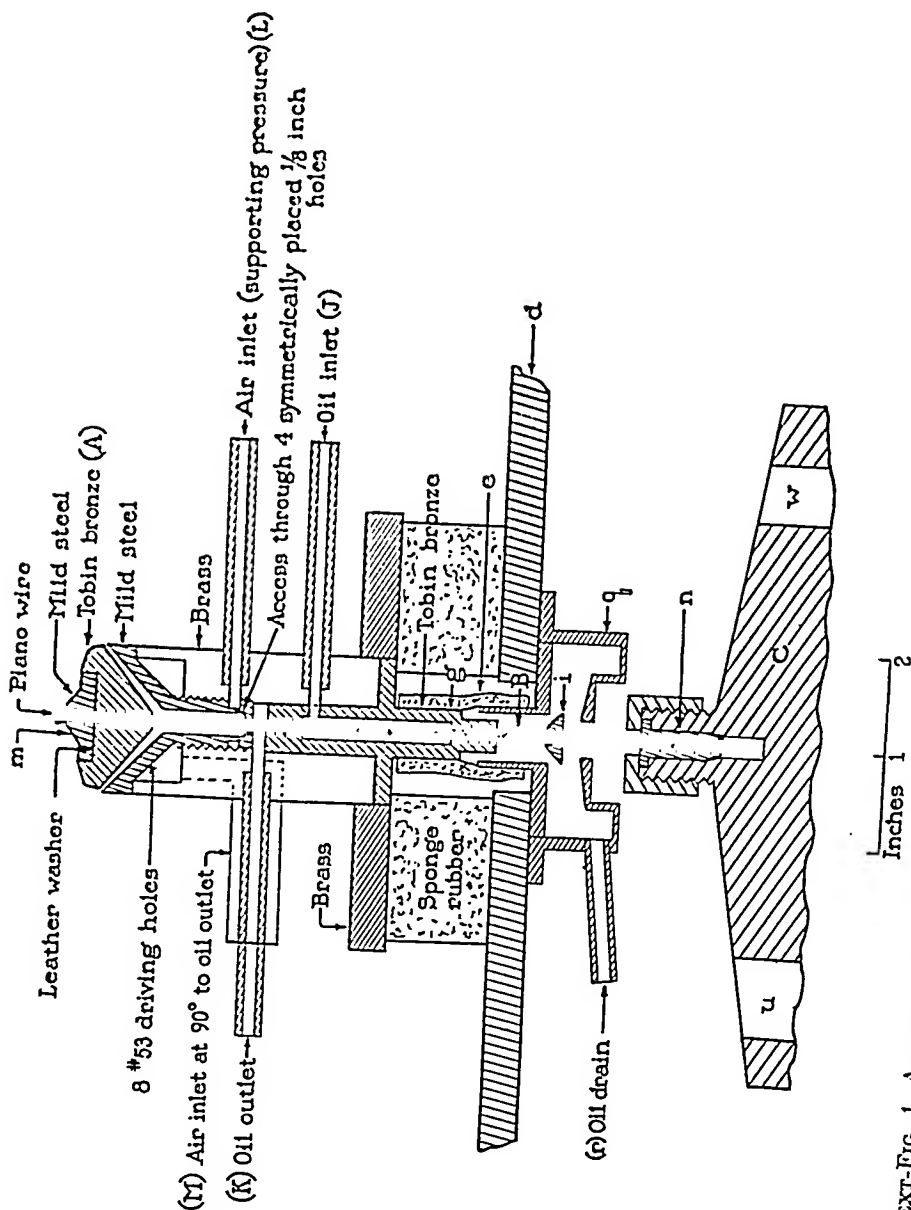
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TEXT-FIG. 1. A cross sectional drawing of the rotating system of the ultracentrifuge. Parts lettered in this and the plates are described in the text.

a greater bearing surface, necessitating a greater driving air pressure and a somewhat increased flow of oil.

The rotors for this ultracentrifuge have thus far been of duralumin. A study⁴ has been made to establish a suitable rotor shape and to determine the maximum speed that can safely be attained with the commercially available alloys. A rotor of the chosen shape is shown at (C). It is 7 inches in diameter and of a tapering thickness that is 1 inch at the edge and 2 inches near the axis; the outside edge of the cell hole is half an inch from the periphery. The maximum permissible speed for such a rotor made of ordinary duralumin (17 ST) is slightly over 800 R.P.S.; if alloy 14 ST is used, 900 R.P.S. are possible. At 800 R.P.S. the field at the center of the cell is 180,000 times gravity; at 900 R.P.S. it is 225,000 gravity. These experiments on rotors have also indicated the amount of shielding that must be provided against bursting rotors. The top and bottom plates (*d*) of the vacuum chamber are of half inch boiler plate, the cylinder (*D*) is of 1½ inch thick heat-treated chrom-vanadium steel. Protection from a broken turbine is furnished by the three sided wooden barricade (*P*) filled with sand.

When in operation a vacuum of less than 1 mm. of mercury is maintained in the chamber (*D*) by a fast oil pump operating through the port (*k*). Residual air pressure is indicated by a gauge (*p*) connecting with (*l*). The cylinder (*D*) and the top and bottom plates (*d*) are sealed by soft rubber rings.

The liquid to be centrifuged is contained in a cell (*u*) that is essentially the same as Svedberg's. It differs mainly in that, being fillable from the end during assembly, an oil seal is unnecessary.

The optical system for absorption measurements of the extent of sedimentation is indicated in Figs. 1, 3, and 4. In our work we are dealing with proteins, all of which show strong absorption for ultraviolet wave lengths shorter than *ca.* 2750 Å. Combined chlorine and bromine filters of appropriate thickness (8 cm. of chlorine gas, 2.5 cm. of bromine vapor at room temperature) absorb all wave lengths between the green and *ca.* 2700 Å. Distribution of protein within the cell can therefore be recorded on photographic plates sensitive only to the blue (and ultraviolet) by employing the light from a quartz mercury lamp after passage through these filters. In Fig. 4 light from the mercury arc in (*E*) is rendered roughly parallel by the quartz condensing lens (*R*) and, passing through the bromine (*S*) and chlorine (*T*) filters, is reflected by the aluminum-sputtered mirror (*V*) through quartz windows into the vacuum chamber (*D*). The light through the cell emerges through a second window (*a*) in the top of the chamber, is reflected by a second aluminum mirror in (*W*) and is focused by the 100 cm. focal length quartz-fluorite lens at (*X*) upon the plate at (*G*). The total distance from the rotor *C* to the plate *G* is 4½ meters; the lens (*X*) can move through 12 cm. about a point in (*F*) 3 meters distant from (*G*). When in operation exposures

⁴ Biscoe, J., Pickels, E. G. and Wyckoff, R. W. G., *Rev. Scient. Instruments*, 1936, in press.

flexible connection between the driver and the top (*d*) of the vacuum chamber is provided by a short length of rubber pressure tubing (*c*). If the rotor is to turn smoothly the bearings must be carefully and accurately reamed to fit closely the chosen shaft; furthermore this shaft should be straight and precisely centered in the turbine clutch (*m*). It is equally important that the rotor clutch (*n*) be exactly in the axis of rotation and that the dummy cell (*w*) be of such a weight that when loaded with its cell the rotor is well balanced about the axis.

The speed of the turbine and rotor is determined by observing with a stroboscope a spot of paint on the top of the turbine. The stroboscope consists of a slotted disc (*Q*) mounted, together with a small magneto, on the shaft of a storage battery driven motor. Speeds can thus be read directly in terms of the voltage developed by the magneto. The necessary compressed air is drawn from the house high pressure lines. A very small flow (a few cubic centimeters per hour) at 5–15 lb. pressure is used to force oil from the tank (*H*) through the inlet (*J*) into the pocket between the bearings. Oil passing the upper bearing drips from the outlet (*K*); that passing the lower bearing is thrown clear of the shaft by a conical deflector (*i*), falls into a well (*g*) and drips through (*r*) into a collector attached to the vacuum chamber. At low speeds it is desirable to use a supporting air column for the rotating system. For this purpose air at 30–40 lb. pressure is introduced through the inlet (*L*); at higher speeds, above *ca.* 200 r.p.s., this air stream is not of advantage. The driving air, at pressures up to *ca.* 100 lb./sq. in., is fed into the inlet (*M*). The pressure on the usual compressed air line is variable so that some kind of regulation is needed to drive the rotor at constant speed. For studies of sedimentation velocity it is possible to obtain a sufficiently good manual control using a well made gate valve as a reducer. It is, however, better to install an automatic pressure regulating reducer for sedimentation rate determination, and one is essential for the long equilibrium runs. Several such reducing valves capable of handling a sufficiently large air flow are made commercially; one is shown at (*N*). At low speeds this regulator results in rotor speeds that are constant within 1 per cent even when the line pressure varies by 40–50 lb./sq. in.; at the highest speeds the regulator has given constancy within *ca.* 2 per cent but this can be improved either by manual help or by having a steadier line pressure. It is very important that the incoming air be clean, both for the proper functioning of such a regulator as (*N*) and to prevent clogging of the driving ports. One of the numerous commercially available compressed air filters should therefore be installed close to the regulator, and brass and rubber rather than iron piping should be used between the filter and the turbine.

The optimum diameter of the steel driving shaft will depend on the speed at which the ultracentrifuge is to operate. For higher speeds (*ca.* 300–900 r.p.s.) piano wire or drill rod 0.100–0.110 inch thick is satisfactory. It is essential that the shaft be strictly uniform in diameter, and straight. While a shaft of this size can be used for speeds below *ca.* 300 r.p.s. the resulting system is liable to show several regions of mechanical vibration when turning slowly. These vibrations disappear if the shaft is of 3/16 inch drill rod. This larger shaft of course presents

a greater bearing surface, necessitating a greater driving air pressure and a somewhat increased flow of oil.

The rotors for this ultracentrifuge have thus far been of duralumin. A study⁴ has been made to establish a suitable rotor shape and to determine the maximum speed that can safely be attained with the commercially available alloys. A rotor of the chosen shape is shown at (C). It is 7 inches in diameter and of a tapering thickness that is 1 inch at the edge and 2 inches near the axis; the outside edge of the cell hole is half an inch from the periphery. The maximum permissible speed for such a rotor made of ordinary duralumin (17 ST) is slightly over 800 R.P.S.; if alloy 14 ST is used, 900 R.P.S. are possible. At 800 R.P.S. the field at the center of the cell is 180,000 times gravity; at 900 R.P.S. it is 225,000 gravity. These experiments on rotors have also indicated the amount of shielding that must be provided against bursting rotors. The top and bottom plates (*d*) of the vacuum chamber are of half inch boiler plate, the cylinder (*D*) is of 1½ inch thick heat-treated chrom-vanadium steel. Protection from a broken turbine is furnished by the three sided wooden barricade (*P*) filled with sand.

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⁴ Biscoe, J., Pickels, E. G. and Wyckoff, R. W. G., *Rev. Scient. Instruments*, 1936, in press.

are made with an electromagnetically operated timing shutter placed between the condensing lens (*R*) and the filter (*S*). A rotating shutter of variable aperture (not shown) is used to lengthen the exposure when making the variously timed exposures required for plate calibration. The exposure varies from 1–10 seconds depending on the type of photographic plate used and the voltage drop across the lamp. With a camera as long as that shown, precautions must be taken to prevent vibration. This has been done by mounting the tube on Lally columns (*Y*₁, *Y*₂, *Y*₃). The bases of these columns, as well as the posts (*Z*) that support the ultracentrifuge, are all imbedded in blocks of cement themselves supported on half inch thick rubber pads.

Provision has been made for simultaneous measurements by the absorption and refractive index methods. To do this a second set of windows (*b*) and a second, and longer, camera tube (*f*) have been placed at right angles to those for absorption.

In order to have accurate measurements of sedimentation it is necessary to know the temperature of the sample during the experiment. There are two ways in which the rotating system could become heated. One is through friction developed in the shaft bearings (*g*), the other is through frictional interaction between the rotor and the small amount of gas remaining in the evacuated chamber. We have made a number of experiments to estimate the amount of this heating. In one set a thermocouple was inserted into the lower bearing (*g*) and a second couple of very low heat capacity was mounted within the chamber along the axis of the turning rotor, and close to it. In other tests the ultracentrifuge was operated at high speed for many hours, stopped as quickly as possible and the rotor temperature compared with that of the chamber walls. Having also determined the rate of cooling of a stationary rotor it was possible to estimate the maximum temperature of the turning rotor. These trials have shown that under our conditions of operation the maximum rise in temperature for runs at 800 R.P.S. is of the order of 2°C.; at lower speeds it is even less. The switchboard used for handling these thermocouple measurements during runs is shown at (*h*).

In order to check the operation of the ultracentrifuge before using it upon proteins of unknown molecular weights, a series of determinations was made of the sedimentation constant of horse hemoglobin that had been repeatedly recrystallized according to the methods of Heidelberger.⁵ One of these photographs made with the rotor turning at 800 R.P.S. is shown in Fig. 5. Sedimentation constants from individual experiments differed from the mean by 0.1×10^{-13} cm./sec. Several systems consisting of viruses and of crystallizable proteins are now being analyzed with this instrument. Results obtained with

⁵ Heidelberger, M., *J. Biol. Chem.*, 1922, 53, 31.

various strains of the crystalline proteins⁴ of the tobacco mosaic disease will shortly be published; one of the photographs from this study illustrating the sedimentation of a large molecule at low speeds is reproduced in Fig. 6.

We are indebted to Mr. J. B. Lagsdin for much help in connection with the design and building of this apparatus.

SUMMARY

Details of construction are given for an air-driven ultracentrifuge for molecular sedimentation. This instrument, like the standard oil-driven machine of Svedberg, uses rotors giving a 6.50 cm. radius of rotation and has cameras of great depth of focus.

EXPLANATION OF PLATES

PLATE 2

FIG. 1. A view of the control panel and the absorption camera of the ultracentrifuge.

FIG. 2. A photograph of the rotating system removed from the vacuum chamber. Cf. Text-fig. 1.

PLATE 3

FIG. 3. A view of the centrifuge and its mountings.

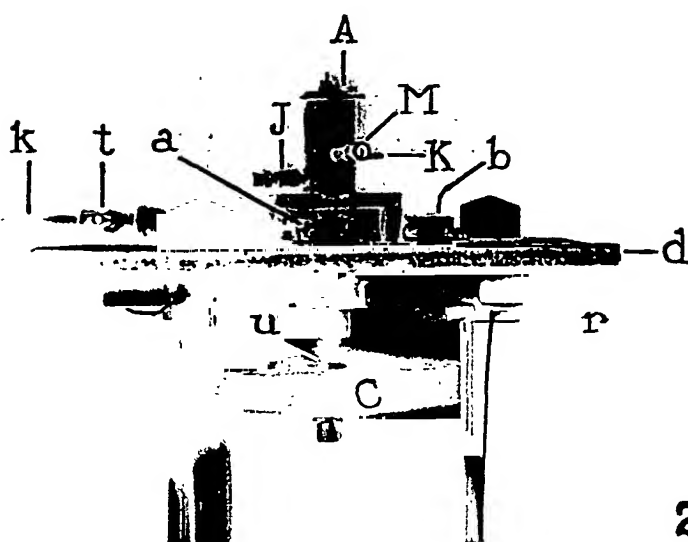
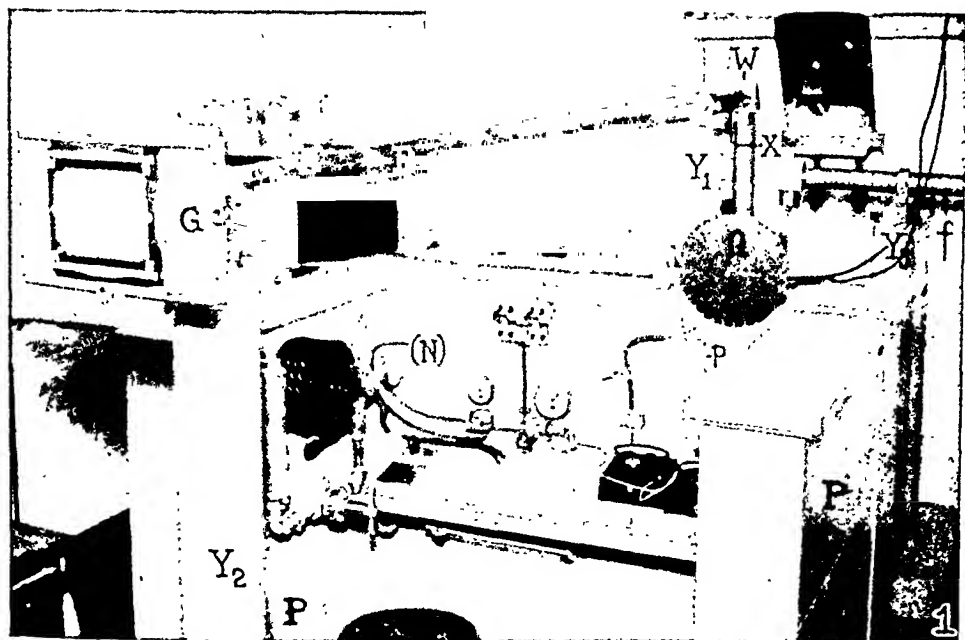
PLATE 4

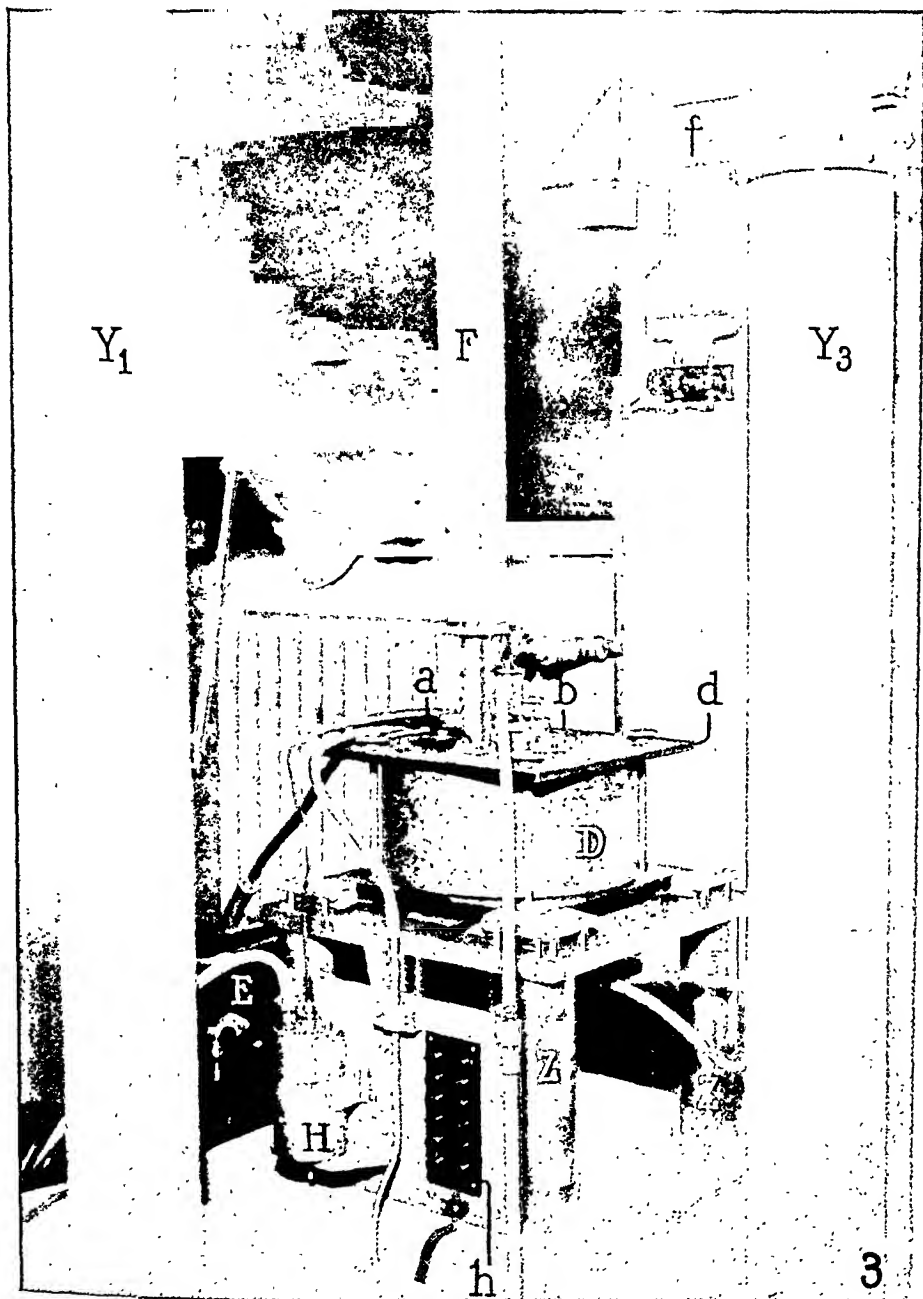
FIG. 4. A view of the optical system used for the ultraviolet photography of proteins by the absorption method.

FIG. 5. A series of pictures showing the course of sedimentation of hemoglobin molecules. Light source: visible light from incandescent lamp. Exposure time: 2 seconds. Speed: 800 R.P.S. Interval between exposures: 10 minutes.

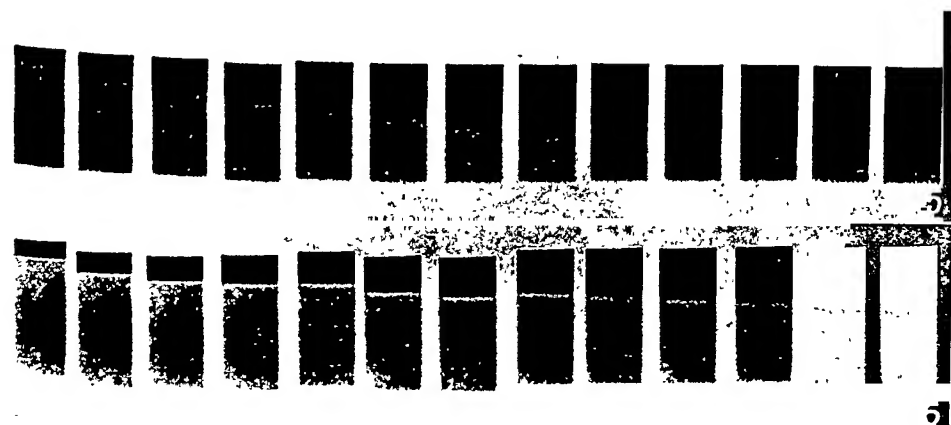
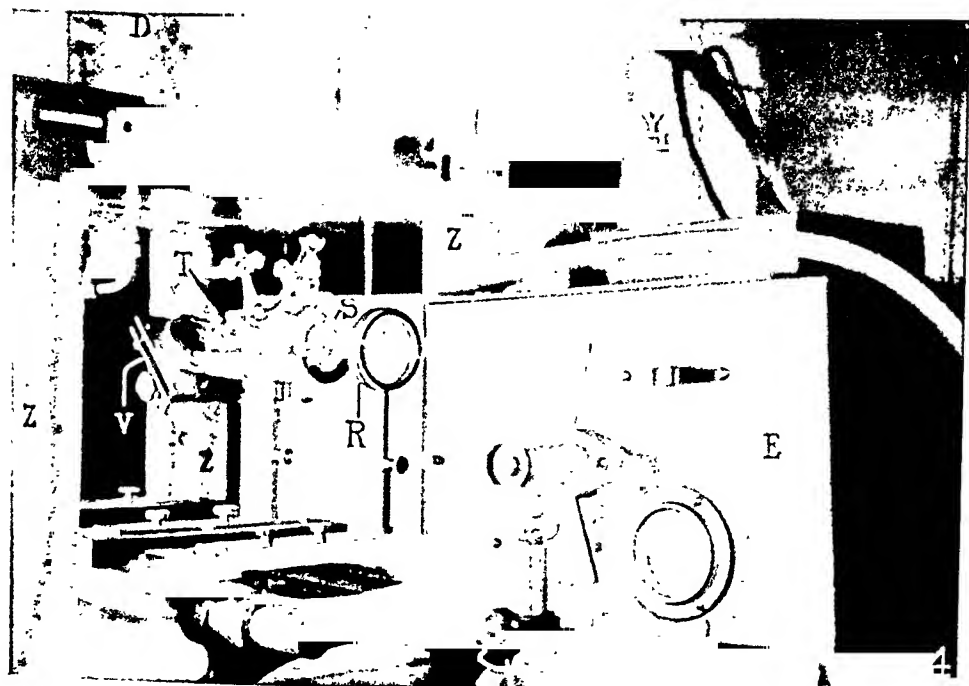
FIG. 6. A similar picture showing sedimentation of the very heavy crystalline protein⁶ of the tobacco mosaic disease. Light source: ultraviolet light after filtration through chlorine and bromine cells. Exposure time: 4 seconds. Speed: 140 R.P.S. Intervals between exposures: 5 minutes.

⁶ Stanley, W. M., *Science*, 1935, 81, 644.









IMMUNIZATION EXPERIMENTS WITH SWINE INFLUENZA VIRUS

By RICHARD E. SHOPE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In earlier experiments (1) it was shown that swine influenza virus, administered intramuscularly, immunized pigs to swine influenza and achieved this result without inducing evidence of infection. It was pointed out that this method of immunization might be of practical value.

The discovery by Smith, Andrewes, and Laidlaw that ferrets (2) and mice (3) are also susceptible to swine influenza virus has made it possible to compare the immunity produced by various methods in these small animals with that similarly produced in the natural host. The present experiments were conducted in an effort to determine the effect of dosage, route of administration, and animal source upon the efficacy of swine influenza virus in immunizing swine, ferrets, and mice.

EXPERIMENTAL

Preparation of Virus for Use as Vaccine

The strain 15 (Iowa, 1930) swine influenza virus was used in all experiments. It will be designated swine, ferret, or mouse virus in this paper to indicate its immediate animal source and the only species other than swine through which it has passed. All mouse virus used had been transferred serially at least five times in mice, and all ferret virus at least fifteen times in ferrets.

To prepare virus for use as vaccine, weighed amounts of infected lung which had been in glycerol in the refrigerator for from 3 days to a month were ground with sand to make 5 per cent suspensions in physiological salt solution. These were allowed to sediment for 10 minutes and the supernatant fluid removed by pipette was employed as the vaccine. All virus suspensions were prepared on the day on which they were to be used.

Titration of Swine Influenza Virus Used to Vaccinate

The approximate number of mouse-infecting doses of virus per cubic centimeter of vaccine was estimated in some of the experiments. While these figures are not exact, they furnish an idea of the relative amounts of virus administered during the period of immunization. They were obtained as follows: Etherized mice were inoculated intranasally as previously described (4) with dilutions of 5 per cent virus suspensions, ranging at intervals of 10, to 1:10,000. 3 or 4 mice were inoculated with each dilution. All mice surviving on the 6th day were killed with chloroform and their lungs, as well as the lungs of those which died earlier, were examined for influenzal lesions. The highest dilution of virus causing definite lung lesions in one or more mice inoculated was taken as the virus titer. From this the number of mouse-infecting doses of virus per cubic centimeter of 5 per cent suspension was calculated on the assumption that approximately 0.1 cc. (4) of suspension entered the respiratory tract of each mouse inoculated. Thus a suspension whose highest infectious dilution was 1:100 would contain 100 mouse-infecting doses of virus for each 0.1 cc. or 1000 per cc. These approximate values are recorded in two of the following tables.

Active Immunization of Swine to Swine Influenza

While it was known that swine virus administered intramuscularly actively immunized swine to swine influenza (1), it seemed of interest to determine whether ferret virus and mouse virus would achieve a similar result.

A number of swine were given two subcutaneous or intramuscular inoculations, 8 days apart, of swine influenza virus from various animal sources. They were tested for immunity to swine influenza 15 or 33 days after their last immunizing dose of virus by the intranasal instillation of a mixture of swine influenza virus and *H. influenzae suis* (5). After a 4 day observation period following the immunity test, during which their temperatures were recorded morning and evening, they were killed by chloroforming or bleeding. Their respiratory tracts were examined at autopsy for lesions of influenza and the lungs, and in some cases turbinates, were tested for virus by inoculation into mice. Blood serum obtained from each pig before and after immunization was tested in mice for virus-neutralizing antibodies by a method already described (6). The results of the immunization experiments in swine are given in Table I.

As shown in Table I, 7 swine which received intramuscular or subcutaneous injections of swine influenza virus from ferrets, mice, or swine were found immune to swine influenza when tested later by intranasal inoculation with a mixture of swine influenza virus and *H. influenzae suis* (5). 2 control swine similarly inoculated developed

TABLE I
The Immunization of Swine to Swine Influenza

Swine No.	Vaccination		Results of tests for immunity			Virus in respiratory tract at postmortem as tested by mouse inoculation						Neutralization of swine influenza virus by swine test					
	Source of virus	No. and route of inoculations (each inoculation 10 cc.)	Interval between last inoculation and immunity test	Clinical illness	Lung lesions at autopsy	Turbينات			Lungs			Virus + serum drawn before immunization			Virus + serum drawn immediately before immunity test		
						Mouse			Mouse			Mouse inoculated with mixture			Mouse inoculated with mixture		
						1	2	3	1	2	3	1	2	3	1	2	3
15-30	Swine	2—intramuscular	days	None	None							4+	4+	4+	0	0	0
15-53	Mouse	2—	33	"	"				0*	0	0	4+	2+	4+	±	1+	0
15-61	Ferret	2—	33	"	"				0	0	0	4+	2+	4+	0	0	0
15-73	Nil (unvaccinated control)			Swine influenza	4 lobes pneumonia							4+	4+	4+	0	0	1+
16-40	Mouse	2—subcutaneous	15	Transient malaise—no temperature elevation	None	0	0	0	0	0	0	4+	4+	4+	0	1+	2+
16-51	"	2—	15	None	"	2+	1+	2+		0	0	4+	4+	4+	0	1+	0
16-52	"	2—intramuscular	15	"	"	0	0	0	0	0	0	4+	3+	4+	1+	0	0
16-58	"	2—	15	"	"	0	0	0	0	0	0	4+	4+	4+	0	0	0
16-62	Nil (unvaccinated control)			Swine influenza	5 lobes pneumonia							4+	4+	4+	0	0	0

* 0 = mouse with no pulmonary lesions at autopsy.

± to 4+ = mice with progressive degrees of influenzal pneumonia; 4+ indicates a complete and fatal pneumonia.

† The swine lung is comprised of 7 lobes.

swine influenza that was typical both clinically and at autopsy. The ferret and mouse viruses appeared to be as effective in immunizing swine as was that derived from swine.

No virus could be demonstrated in the lungs of the 6 swine tested although it was found in the turbinates of one of them. Previous experiments have shown that virus is regularly and abundantly present in the turbinates, tracheal exudate, and lungs of susceptible swine killed on the 3rd or 4th day of an influenza infection (7). The immunized animals were thus not only refractory to infection but had also, with one exception, inactivated or destroyed the virus administered in testing for immunity. In the exceptional animal, virus established itself in the nose but failed to invade the lung.

Antibodies neutralizing swine influenza virus appeared in the sera of all animals during the course of immunization. It was estimated, without recourse to titration, that these were of lower titer than those resulting from an attack of the disease.

Active Immunization of Ferrets

Smith, Andrewes, and Laidlaw (8) attempted to immunize ferrets to swine and to human influenza virus by repeated subcutaneous injections of each virus. According to a personal communication, ferrets were the immediate animal source of the virus used. Of the 11 ferrets included in their experiments, 2 were found completely resistant later to the test dose of virus given by intranasal or intrapulmonary inoculation under ether narcosis. The remaining animals developed either nasal symptoms or fever much like the controls. They differed from the controls, however, in that they showed no lung lesions at autopsy. It was concluded that in these animals a partial immunity, sufficient to protect the lungs from virus attack, had been established.

In the present experiments an attempt was made to immunize ferrets to swine influenza virus by the subcutaneous or intraperitoneal injection of ferret, mouse, or swine virus.

2 cc. doses of 5 per cent infected lung suspension were administered either once or twice, at 8 day intervals, to each ferret. The animals were tested for immunity, 15 to 41 days after their last immunizing injection, by intranasal inoculation under ether narcosis with 1 cc. of a 5 per cent suspension of swine influenza virus derived from ferret lung. After an observation period of from 4 to 7 days following the

TABLE II
The Immunization of Ferrets to Swine Influenza Virus

Ferret No.	Vaccination				Results of tests for immunity		Virus in respiratory tract at postmortem as tested by mouse inoculation					
	Source of virus	No. and route of inoculations (each inoculation 1 cc.)	Mice infected (no. of virus per cc.)	Interval between last inoculation and immunity test	Clinical illness	Lung lesions at autopsy	Turbinate			Lung		
							Mouse			Mouse		
							1	2	3	1	2	3
4-6	Ferret	2—subcutaneous		days								
4-9	"	2—"		15	0*	0†						
5-7	"	2—"		15	0	0						
5-8	"	2—"		15	0	0						
7-3	"	2—"		15	0	0	0†	0	0			
5-3	"	2—"	1000	33	0	0	0	0	0			
5-5	"	1—"		15	0	0						
6-0	"	1—"		23	0	0	0	0	0			
8-0	"	1—"		23	0	0	0	0	0			
5-6	Swine	2—"	1000	33	++	++						
6-1	"	2—"		15	++	+						
8-2	"	2—"		15	+++	+++						
7-9	"	1—"	1000	33	++	+++						
8-3	"	1—"	1000	41	++	+++						
9-3	"	2—"	1000	41	++	++						
9-0	"	2—intraperitoneal	1000	15	++	++	4+	4+	3+	2+	2+	2+
9-1	"	2—"	1000	15	+	+++	4+	4+	2+	3+	2+	3+
9-2	"	2—"	1000	15	0	0	4+	3+	3+	0	0	0
			1000	15	0	0	4+	3+	2+	0	0	0

* Clinical illness:

0 = none.

+ = clinical picture that of mild influenza.

++ = an influenza of average severity.

+++ = severe. Most of the ferrets with this degree of illness would probably have died.

† Lung lesions:

0 = none detectable at autopsy.

+ = influenzal pneumonia involving less than $\frac{1}{4}$ of lung at postmortem.

++ = influenzal pneumonia involving from $\frac{1}{4}$ to $\frac{1}{2}$ of lung at postmortem.

+++ = influenzal pneumonia involving from $\frac{1}{2}$ to $\frac{3}{4}$ of lung at postmortem.

‡ Mouse inoculations: 0 = no pulmonary lesions at autopsy.

± to 4+ = progressive degrees of influenzal pneumonia.

4+ indicates a complete and fatal pneumonia.

TABLE II—*Concluded*

Ferret No.	Vaccination				Results of tests for immunity		Virus in respiratory tract at postmortem as tested by mouse inoculation					
	Source of virus	No. and route of inoculations (each inoculation 2 cc.)	Mouse-infecting doses of virus per cc.	Interval between last inoculation and immunity test	Clinical illness	Lung lesions at autopsy	Turbinates			Lung		
							Mouse			Mouse		
							1	2	3	1	2	3
				days								
7-5	Mouse	2—subcutaneous	1000	33	+	+						
7-6	"	2—"	1000	33	+	+						
7-7	"	1—"	1000	41	++	++						
8-1	"	1—"	1000	41	++	+++						
9-7	"	2—"	10,000	15	++	0	1+	2+	2+	0	0	0
9-4	"	2—intraperitoneal	10,000	15	0	0	1+	1+	±	0	0	0
9-5	"	2—"	10,000	15	0	0	0	0	0	0	0	0
9-6	"	2—"	10,000	15	0	0	0	0	0	0	0	0
4-8	Nil (unvaccinated control)				+++	+++						
6-2	"	"	"	"	++	++						
7-8	"	"	"	"	++	+++						
8-4	"	"	"	"	+++	+++						
8-7	"	"	"	"	+++	+++						
9-9	"	"	"	"	++	+++	3+	2+	2+	4+	4+	3+
10-0	"	"	"	"	++	++	4+	2+	2+	4+	2+	3+
10-1	"	"	"	"	++	++	3+	3+	2+	4+	4+	3+

immunity test, during which their temperatures were recorded morning and evening, they were killed by chloroforming. Their respiratory tracts were examined at autopsy for evidence of infection (9) and in some cases the turbinates and lungs were tested for virus by inoculation into mice. The results of attempts to immunize ferrets to swine influenza virus are given in Table II. The experiments included were not all conducted simultaneously.

As shown in Table II, 8 of 9 ferrets that had received one or two subcutaneous injections of ferret virus were rendered immune to swine influenza virus. Little if any immunity, however, was established by the similar administration of swine or mouse virus. 6 ferrets that had received either one or two injections of swine virus, and 4 ferrets that had received one or two injections of mouse virus subcutaneously, were not immune and differed little or not at all from the control

animals with respect to illness and lung lesions exhibited following their test infection. One ferret (No. 9-7) that had received two subcutaneous injections of mouse virus developed an influenza that appeared typical clinically, but at autopsy its lungs were normal. It is apparent from the above experiments that, when given subcutaneously, ferret virus is superior to that from either mice or swine in immunizing ferrets to swine influenza virus. No reason for this superiority of homologous over heterologous virus is evident.

The advantage of ferret over swine or mouse virus was less apparent when the immunizing inoculations were given intraperitoneally. All 3 ferrets that had received mouse virus into the peritoneal cavity and 2 of 3 of those similarly inoculated with swine virus were rendered clinically immune to swine influenza virus. These experiments indicate that the route by which heterologous swine influenza virus is administered to ferrets determines, to a marked degree, its effectiveness in producing immunity.

The results of the tests for virus in the turbinates and lungs of a number of the ferrets, given in the last column of Table II, indicate that the lungs of immunized animals, which appeared normal at autopsy, were also free from detectable virus. However, the turbinates of some of the ferrets that had shown no clinical symptoms contained sufficient virus to infect mice. It is probable that these ferrets had been less effectively protected than those in which virus failed to become established in the turbinates following the test for immunity.

The 14 ferrets in Table II which showed varying degrees of immunity may be grouped into three classes: those immune and free from demonstrable virus; those immune which had virus in the turbinates, and the single ferret (No. 9-7) which, though not clinically immune, developed no lung lesions and had virus only in its turbinates. The majority of ferrets in the experiments reported by Smith, Andrewes, and Laidlaw (8) would belong in the last group.

Active Immunization of Mice

Smith, Andrewes, and Laidlaw (8), and Francis and Magill (10) have reported the immunization of mice to human influenza virus by means of repeated doses of virus given subcutaneously, intradermally, or intraperitoneally, or by a combination of these routes.

The following experiments were conducted in an effort to define the conditions required for the immunization of mice to swine influenza virus. Preliminary experiments had suggested that mice behaved towards homologous and heterologous swine influenza virus much as did the ferrets described in the preceding section. It seemed likely, therefore, that the question of immunization with swine influenza virus from various animal sources could be investigated more thoroughly in mice than in ferrets. Moreover, since the infection produced by swine influenza virus in mice is both highly fatal and noncontagious (4), the efficacy of immunization procedures in this species may be determined by survival alone and the extreme isolation precautions essential with ferrets or swine are unnecessary.

Mice 3 to 5 weeks old and weighing from 10 to 15 gm. at the beginning of the immunization procedure were used. 0.2 cc. doses of 5 per cent infected lung suspension were administered, either once, or repeatedly at 8 day intervals, to each mouse subcutaneously or intraperitoneally as recorded in Table III. The animals were tested for immunity to mouse lung swine influenza virus (either a 2 per cent or 5 per cent suspension) administered intranasally under ether narcosis (4) 14 or 30 days after their last immunizing dose of virus. The control mice, acquired from stock at the same time as those to be vaccinated and kept in the same isolation room, quite regularly succumbed to this amount of virus within 7 days. All mice dying were autopsied in order to establish that death had been the result of an influenza virus pneumonia. Survival was taken as the criterion of immunity. The results of attempts to immunize mice to swine influenza virus by various procedures are recorded in Table III.

The four experiments presented in Table III are not strictly comparable for, while the amount of infected lung suspension used to vaccinate was kept constant, the virus content of these suspensions varied from approximately 100 to 10,000 mouse infecting doses per cc. Within individual experiments, however, the results reflect quite clearly the effectiveness of one immunization procedure as compared with others in the same experiment, and even between experiments certain broad comparisons can be made. Of 83 control mice infected in the four experiments, 79 died, indicating the virulence of the virus and the severity of the test for immunity.

Swine virus administered subcutaneously was definitely the least effective of any of the immunization procedures tried; only 5 of 63 mice (8 per cent) thus treated survived the test dose of virus and these 5 survivors were all in Experiment 4 in which an unusually virus-rich

TABLE III

The Immunization of Mice to Swine Influenza Virus

Experiment No.	Vaccination				Results of tests for immunity
	Source of virus	No. and route of inoculations (each inoculation 0.2 cc.)	Mice infecting doses of virus per cc.	Interval between last inoculation and immunity test	
1				days	
	Swine	2—subcutaneous	1000	30	0/19*
	Ferret	2—	1000	30	3/18
	"	2—intraperitoneal	1000	30	8/10
	Mouse	2—subcutaneous	1000	30	6/12
	"	1—	1000	30 and 38	9/19
	"	2—	100	30	3/16
	(diluted 1:10)				
2	Mice recovered from intranasal infection with swine virus				8/8
	Unvaccinated control mice				0/19
3	Swine	2—intraperitoneal	100	14	11/18
	Ferret	2—	100	14	12/20
	Mouse	2—	1000	14	10/18
	"	2—	100	14	11/20
	(diluted 1:10)				
4	Mice recovered from intranasal infection with swine virus				6/6
	Unvaccinated control mice				1/20
	Swine	3—subcutaneous	100	14	0/20
	"	3—intraperitoneal	100	14	8/18
5	Mouse	3—subcutaneous	100	14	11/14
	"	3—intraperitoneal	100	14	10/11
	Unvaccinated control mice				2/20
	Swine	3—subcutaneous	10,000	14	5/24
	"	3—intraperitoneal	10,000	14	23/24
	Mouse	3—subcutaneous	10,000	14	19/23
	"	3—intraperitoneal	10,000	14	21/21
	"	1—subcutaneous	10,000	30	11/24
	"	1—intraperitoneal	10,000	30	12/25
6	"	3—subcutaneous (with 10 per cent swine serum)	10,000	14	16/18
	"	3—intraperitoneal (with 10 per cent swine serum)	10,000	14	21/23
	"	3—subcutaneous (diluted 1:10)	1000	14	15/24
	"	3—intraperitoneal (diluted 1:10)	1000	14	13/23
	Unvaccinated control mice				1/24

* The numerator represents the number of mice that survived the immunity test; the denominator the number of mice in the group tested.

vaccine had been employed. Ferret virus given subcutaneously also failed to induce an appreciable degree of immunity.

Swine virus given intraperitoneally, on the other hand, produced a fair degree of immunity; 42 of 60 mice (70 per cent) thus treated survived the test dose of virus. In Experiment 4, in which a swine lung vaccine rich in virus had been used, 23 of 24 mice survived. Ferret virus was also a better immunizing agent when given intraperitoneally, 20 of 30 mice (66 per cent) thus treated surviving the immunity test.

Mouse virus administered two or three times proved the best immunizing agent for mice and the intraperitoneal route held only a slight advantage over the subcutaneous route. 36 of 49 mice (73 per cent) that had received mouse virus subcutaneously and 41 of 50 mice (82 per cent) that had received it intraperitoneally survived the test infection. Single injections of mouse virus given either subcutaneously or intraperitoneally produced an immunity that was inferior to that following multiple injections. Only 17 of 43 mice (40 per cent) that had received a single subcutaneous dose of mouse virus and 12 of 25 mice (48 per cent) that had received a single intraperitoneal injection survived the test dose of virus. The importance of dosage of virus administered in establishing immunity is indicated by the two groups of mice receiving multiple inoculations of 0.2 cc. amounts of 0.5 per cent instead of the usual 5 per cent mouse virus. Only 18 of 40 mice (45 per cent) receiving multiple injections of this dilute virus subcutaneously and 24 of 43 mice (56 per cent) receiving it intraperitoneally survived the test dose of virus. From this it would appear that multiple injections of 0.5 per cent mouse virus were only slightly, if at all, superior to single injections of 5 per cent mouse virus in immunizing mice.

Laidlaw and Dunkin (11) suggested that the multiplicity of antigens contained in heterologous dog distemper vaccine interfered with the antibody response to formolized virus and thus accounted for its inability to immunize. It seemed possible that this explanation might also account for the failure of swine virus given subcutaneously to immunize mice to swine influenza virus. However, the addition of normal swine serum to mouse virus did not appreciably alter its capacity to immunize mice (Experiment 4 of Table III), suggesting that some more complex explanation was applicable here.

Risk of Infection during Immunization with Swine Influenza Virus

A small number of mice succumbed during the period they were receiving their immunizing injections of swine influenza virus. These were carefully autopsied in an effort to determine the cause of death. In most instances intestinal infections with an accompanying diarrhea were responsible. In a few, however, pneumonia was encountered. The lungs of such animals were tested for the presence of swine influenza virus by mouse inoculation, but in no instance was it demonstrated. None of the ferrets or swine became ill during the course of immunization and their temperatures, recorded daily, remained within normal limits. The present experiments thus afford no evidence that the administration of swine influenza virus subcutaneously, intraperitoneally, or intramuscularly, entails any risk of infection.

Experience in some unpublished immunization experiments conducted among swine on farms in eastern Iowa, however, suggests that under certain conditions immunization with swine influenza virus may be a hazardous procedure.

In the field experiments referred to, 1635 swine on 55 different Iowa farms were given one or more intramuscular injections of glycerolated swine influenza virus. 3603 other swine on these same farms were left uninoculated to serve as controls should an epizootic of swine influenza later appear. Wherever feasible the vaccinated swine were kept isolated from the remainder of the drove for a period of from 10 days to 2 weeks. In a number of instances, however, there were no facilities for isolation and it was necessary to keep the inoculated swine in the same yards with uninoculated animals. In two such droves swine influenza appeared shortly after swine influenza virus had been administered intramuscularly to a portion of the animals.

Drove 1 contained 223 swine. Early in August, 12 days after 23 of these animals had received an intramuscular injection of swine influenza virus, swine influenza appeared in the drove. On the 4th day following onset all save 30 animals were typically ill of influenza. Among these 30 apparently normal swine were 20 of the 23 that had received virus intramuscularly 16 days earlier. So far as could be determined, there was, at the time, no other swine influenza in eastern Iowa to which this outbreak could be traced. Furthermore, it was early August, fully 2 months before swine influenza ordinarily becomes prevalent in the Middle West. The length of time (12 days) elapsing between vaccination and the appearance of disease in the swine eliminated from consideration the possibility that they had become infected by virus accidentally spilled in the yards at the time of vaccination. The most probable source of infection seemed to be the animals to

which swine influenza virus had been administered intramuscularly. It is believed, although it cannot be proved from the data at hand, that virus spread from the intramuscular site of inoculation and invaded the respiratory tract of one or more of these animals. From here it was transmitted rapidly by contact among the 200 susceptible swine in the drove. Either the swine first infected, or some of those to which the virus was transmitted very early, must have been carriers of *H. influenzae suis* for the disease developing in the drove was swine influenza (caused by the combined action of virus and *H. influenzae suis* (5)), and *H. influenzae suis* was recovered from the pneumonic lung of one of the fatal cases. 20 of the 23 vaccinated animals failed to develop influenza at the time the remainder of the herd became ill, probably because the 12 days elapsing between their inoculation and the outbreak of the disease had been sufficient for the establishment of immunity. There is considerable likelihood, based on experience with droves in which inoculated animals were kept isolated for 2 weeks after vaccination, that had the 23 vaccinated animals in this herd been kept separate from the 200 non-inoculated swine, no illness would have appeared in either group of animals.

The second drove, in which influenza appeared shortly following the intramuscular administration of swine influenza virus, contained 195 swine. 4 days after 95 of these animals had been vaccinated, influenza appeared in the drove. So far as could be observed all animals became ill. The source of infection is believed similar to that in drove 1, although here the interval between injection and onset of illness was so short that infection from premises contaminated with virus at the time the animals were inoculated could not be eliminated. Insufficient time had elapsed for the development of immunity in the vaccinated animals although the owner was of the opinion that the first cases appeared in unvaccinated swine. As in the case of drove 1, this outbreak occurred in August, but a year later, and it could not be traced to an outside source of infection.

The swine influenza in the two herds just discussed is believed to have been caused by the virus used to vaccinate. The examples cited are considered illustrative of the hazard entailed in the introduction of a "live" virus vaccine into only a portion of a densely crowded susceptible population. To judge from the laboratory experiments with mice, ferrets, and swine and the field experiments with swine, the use of "live" swine influenza virus as a prophylactic agent may be less dangerous to the recipient of the virus than it is to other susceptible individuals with which the recipient may come in contact during the course of immunization.

DISCUSSION

The immunization experiments described indicate that ferrets and mice are similar in their reactions towards swine influenza virus ad-

ministered as a vaccine. With both species only the homologous virus proved an effective immunizing agent when given subcutaneously; ferret or swine virus given by this route to mice, and mouse or swine virus similarly administered to ferrets, established little or no active immunity. These failures were not due to inability of swine influenza virus to immunize when introduced into subcutaneous tissues, because the homologous virus, given by this route, proved effective in both ferrets and mice. Neither were they entirely the fault of the virus suspensions employed because the heterologous virus immunized almost as well as the homologous when given intraperitoneally.

In the case of swine the route of inoculation or the source of the virus used to vaccinate was of little importance, for active immunity followed subcutaneous or intramuscular injection of either homologous or heterologous virus.

The mechanism whereby swine influenza virus, introduced intraperitoneally, intramuscularly, or subcutaneously succeeds in establishing an immunity capable of protecting the highly susceptible tissues of the respiratory tract is unknown. Specific virus-neutralizing antibodies resulting from vaccination may contribute to the immunity, although they can scarcely be held entirely responsible since their presence is not necessarily synonymous with complete active immunity, as shown by Smith, Andrewes, and Laidlaw (8). A possibility which may be entertained only to be discarded is that virus spreads from the site of inoculation to the respiratory tract in minute quantities insufficient to produce clinically recognizable disease but resulting in subclinical infections and subsequent immunity. Against this possibility are two observations brought out in the present experiments: the superiority, as immunizing procedures, of multiple over single virus injections, and the superiority of homologous over heterologous virus given subcutaneously. If immunity were merely the result of subclinical infection, it is not apparent why it should be greatly influenced by number of injections, route of inoculation, or animal source of virus administered. The above arguments are effective in the cases of the mouse and the ferret. They may not, however, apply to swine for, with this species, virus from any susceptible host administered either subcutaneously or intramuscularly confers immunity and the evidence of earlier experiments (1) indicates that a

single intramuscular injection of virus is sufficient to immunize effectively. In spite of the absence of good evidence to the contrary, there is little to indicate that swine influenza virus given intramuscularly to swine, regularly induces immunity by virtue of its invasion of the respiratory tract and its establishment there of a low grade and unrecognized infection. The two droves of swine mentioned, in which influenza appeared shortly after virus had been administered intramuscularly, probably acquired their infections from virus used in the attempted immunization. They thus afford evidence that under certain conditions the virus may spread to the respiratory tract. However, they probably represent exceptional instances, because none of the swine investigated under laboratory conditions showed evidence of illness during immunization and over 1500 animals vaccinated in field experiments remained normal. It thus seems likely that swine, as well as ferrets and mice, can acquire an immunity to swine influenza virus following its administration by unusual routes, without the actual infection of tissues in which it causes disease manifestations.

SUMMARY

1. Swine influenza virus obtained from the lungs of infected ferrets or mice, when administered intramuscularly or subcutaneously, immunizes swine to swine influenza.
2. Ferrets, which have received subcutaneous injections of swine influenza virus obtained from the lungs of infected ferrets, are immune to intranasal infection with this virus. Similar injections with virus from the lungs of infected mice or swine do not immunize.
3. Mice can be immunized to intranasal infection with swine influenza virus by the subcutaneous injection of virus obtained from the lungs of infected mice, but not by similar injection with virus from the lungs of infected ferrets or swine. Repeated injections induce greater immunity than a single one.
4. Intraperitoneal inoculation of both mice and ferrets with swine influenza virus immunizes them to intranasal infection and it appears to make little or no difference whether the virus used as vaccine is obtained from the lungs of infected mice, ferrets, or swine.
5. Field experiments in which swine influenza followed the intramuscular administration of virus are cited as examples of the hazard

involved in the use of this means of immunization in a densely crowded susceptible population.

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SEROLOGICAL REACTIONS WITH A VIRUS CAUSING RABBIT PAPILLOMAS WHICH BECOME CANCEROUS

I. TESTS OF THE BLOOD OF ANIMALS CARRYING THE PAPILLOMA

By JOHN G. KIDD, M.D., J. W. BEARD, M.D., AND PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 5

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The cutaneous papillomas induced in domestic rabbits with a virus procured from western cottontails (1) have the immediate attributes of tumors (2), and they frequently become cancerous (3). The problem of the relation of the virus to the neoplastic activities is complicated by a singular difficulty. Though readily obtained from most of the naturally occurring papillomas of cottontails, it cannot ordinarily be recovered from the far more vigorous growths induced with it in domestic rabbits. Recently indeed, Shope (4) has secured virus strains with which the disease can be serially transmitted in such animals, and it should be possible to make direct tests for the presence of these in any cancers that may develop as result of their action. But the large material thus far studied by us,—comprising more than 150 instances of malignant change in all,—has been provided by the inoculation of domestic rabbits with virus strains irrecoverable from either the papillomas or the cancers, by any of the various means thus far employed. For this reason recourse has been had to a serological method to determine the presence of the virus.

Shope noted that rabbits carrying the papilloma proved more or less resistant on reinoculation, and that their sera exerted a neutralizing influence when mixed with the virus *in vitro* (1). We have titrated the antiviral power, investigated the conditions of its development, and sought for it in the blood of animals carrying tumors of various sorts. The findings will be recorded in two associated papers.

General Method

A method whereby sera could be compared was worked out first.

The virus acts only where brought in contact with injured epidermis, but it is capable of surviving for long periods outside of the body. When many neighboring areas of scarified skin are inoculated they must be protected for a few days, until healing has taken place, in order to exclude all risk of the transfer of virus material from one site to another. This can be readily accomplished by shaving only the immediate areas utilized, placing on each after inoculation a sterile gauze square which fits neatly amidst the fur, mooring these firmly in place with adhesive, and covering all with a large gauze dressing and a many-tailed bandage. In no instance amongst hundreds of tests carried out in this way, often involving more than a score of inoculations into each animal, has a papilloma developed under conditions suggesting that the virus had undergone accidental transfer.

Each inoculation area was a square 3 to 4 cm. across, separated from its neighbors by a furry zone 1 cm. or more wide. It was soaped and shaved, rinsed well in running tap water, and allowed to dry for several hours at room temperature. On the ventral surface of an adult domestic rabbit there is room for two rows of five or six squares, to either side of the midline, and for an additional row of four or five squares immediately next them on each side (Fig. 1). The skin higher on the sides is somewhat thicker and more difficult to scarify, and its fur soon grows again. Hence it is less favorable, both to infection with the virus and to visibility of the young papillomas. Differences in situation as possibly affecting the results were excluded by inoculating the test mixtures and their controls at corresponding places on the right and left, and systematically varying the situations of the inocula from animal to animal.

A strip of sterile sandpaper that could be shifted within a clamp on a handle was used for scarification, with fresh sandpaper for each square. Sterile, dry, Perfection glass test tubes were employed to rub the test mixtures in, each scarified area receiving 3 drops of one of these before the next area was abraded. As soon as all the squares had been inoculated a gentle blast of air warmed by an electric coil was directed upon them to dry them, and then they were dressed as already described. Each animal received all the inocula before the next was dealt with. 2 to 6 rabbits were employed for the tests of every experiment.

The dressings were removed when healing was complete, after 7 to 10 days. The papillomas usually began to appear within 2 to 3 weeks, and their number, character, and rate of development were recorded at intervals of 3 to 5 days until it was plain that no further, pertinent data could be obtained. Fig. 1 shows the range of lesions encountered, from solitary discrete growths to confluent

papillomatosis, the latter being consequent on the action of the virus upon many adjacent cells. Confluent growths could be perceived sooner than scattered ones, and often some of the latter appeared 1 or 2 weeks later than others of the same inoculated square; but skin that was still negative after 6 weeks invariably remained so. The findings were recorded with the aid of the following symbols: +, one or two discrete papillomas; ++, a small number of discrete papillomas; +++, many discrete papillomas; +++, semiconfluent papillomatosis; +++++, confluent papillomatosis. Different observers were found to record the lesions identically, yet throughout the testing the same individual did this work. The recording system made no provision for the rate of enlargement of the lesions, nor for their ultimate fate, but these later phenomena were largely determined by the character of the individual test rabbits, and primary virus neutralization was the subject under study.

Results of Diluting the Virus

On several occasions a virus-containing extract of the glycerinated tissue of a "spontaneous" papilloma has been diluted with Tyrode in multiples of ten, and inoculated into rabbits according to the method described. The 10 per cent extract and the 1 per cent usually gave rise to confluent or semiconfluent proliferation, whereas the higher dilutions yielded scattered papillomas in numbers roughly proportionate to the decrease in virus amount. The most active materials gave rise to only 1 or 2 discrete growths at a dilution of 1 in 100,000, and none was ever obtained at 1 in 1,000,000. Numerous tests carried out incidentally to other experiments have yielded like findings. The virus always acted within a few weeks or not at all. Many of the animals receiving titration mixtures and kept for more than a year have been repeatedly examined with this point in view. The brief delay in the appearance of some of the discrete papillomas of punctate origin can be accounted for by virus infection of but a few cells or a single cell; for the papilloma enlarges by intrinsic proliferation, and the time required for it to become visible will inevitably vary with the number of cells from which it derives. The late-appearing papillomas differed in no essential respect from the generality.

Some areas that remained negative after inoculation with virus dilutions that might conceivably have yielded growths were tarred or injected with Scharlach R in olive oil, and others were painted repeatedly with xylol, to further the action of virus that might have lain latent in the absence of such stimulation. No growths ever developed.

In other experiments having the same object, areas about 12 by 4 cm. in diameter on the sides of susceptible rabbits were infiltrated with active virus extract, by intradermal injections about 2 cm. apart, all at the same level; and subsequently at intervals of 2 weeks the infiltrated skin was traumatized by tattooing to provide the injury necessary to render the virus effective. Again no growths were got save at the points, marked with dye, where the injecting needle had originally been thrust in, and at points where tattooing was done on the day of injection. From all this it seems certain that virus experimentally introduced into the skin of domestic rabbits does not ordinarily lie latent there. It certainly does so on occasion in the cottontail, its natural host. In an animal of this sort, tarred repeatedly on the ears, numerous characteristic, pigmented growths appeared at 2 out of 3 sites on the sides where virus had been inoculated many months previously with negative results. No tar was put on the sides at any time, nor was there any sign there of the diffuse hyperkeratosis consequent on tarring.

Neutralization Tests with the Sera of Domestic Rabbits Bearing Papillomas

General Technique.—Blood specimens were taken from an ear vein into tubes coated with paraffin to prevent hemolysis, and the serum was taken off 24 to 48 hours later and centrifuged to remove the cells. The clot had stood several hours at room temperature and for the rest of the time in the ice box.

The virus materials utilized for the work reported in our previous papers regularly gave rise to growths that enlarged progressively, during the first weeks at least. To broaden the observations it was desirable to employ a material which caused papillomas that retrogressed not infrequently yet which was carcinogenic under favorable conditions. Fortunately one was available, the papillomatous tissue of W.R. 6-32, which had been hashed fine and stored in 50 per cent glycerine. When a fluid of standard virus strength was desired, 4 cc. of this glycerinated tissue was thrown down in a graduated centrifuge tube by spinning at a fixed speed; the amount of sediment was read off; it was suspended in 8 parts of Tyrode solution; thrown down again, ground with sand, made to the required dilution by bulk, usually 6 per cent; and finally spun long at high speed. The central portion of the fluid column was drawn off through a needle, spun again, and its central portion aspirated in the same way. The "departiculated" test material thus obtained was invariably clear, and it had always the same power to produce papillomas, as shown by dilution tests. Such tests were carried out a few days prior to the experiments and again 10 weeks later, that is to say 2 weeks

before the final serum examination. On both occasions virus dilutions of 10^{-4} gave rise to a few growths scattered on the area of inoculation, but 10^{-5} to none. Two mixtures were made of virus with each serum in differing proportions. After incubation at 37°C . for 2 or 4 hours all were inoculated into 3 to 6 normal brown-gray rabbits shaved in squares, with corresponding virus-Tyrodé controls. Some of these rabbits proved more favorable than others, their papillomas appearing sooner and growing more vigorously. The variety thus introduced into the findings did not alter their implications, since each animal served as its own control.

The experiment which follows was devised to test whether the power of the blood to neutralize virus varies with the amount of papillomatous tissue developing. Incidentally an effort was made to determine whether the presence of large growths had any influence on the course of small ones appearing somewhat later.

Experiment 1.—8 brown-gray rabbits of from 1940 to 2850 gm. were paired according to size, weight, and similarity of skin texture. All were bled 5 cc. for serum, and then 10 per cent virus was tattooed into areas 2 mm. across at 3 widely separate points at the same horizontal level on each side. An electric tattooing machine was used. In addition the animals of one group were inoculated broadcast over an abdominal area about 12 by 8 cm.

The growths developing on the sides were traced and charted at intervals of a few days, and the character of the abdominal growths was also recorded. On the 33rd, 42nd, and 68th days after inoculation serum was again procured, and on the last of these days 4 of the rabbits, of differing antiviral power at the most recent test, were reinoculated with two different strains of active 10 per cent virus (W.R. 18 and W.R. 8-76), into scarified areas about 10 cm. across on the sides.

In Chart 1 the skin areas covered by the growths resulting from the tattoo inoculations are shown to scale. The confluent abdominal masses occupied the entire inoculation area in every case, but their heights are indicated diagrammatically by groups of vertical lines joined at the base. The antiviral power of the serum of the host, as expressed in terms of its power to neutralize, is given in hatched columns. An arrow surmounting a column means that neutralization of the virus suspension employed was complete, and hence that the serum titer was probably greater than is shown. The positive or negative outcome of the reinoculations on the 68th day is recorded as R + or R -.

For the titrations just prior to inoculation, portions of the individual sera were mixed in equal parts with 10 per cent virus and 1 per cent virus respectively; for the tests on the 33rd and 42nd days $6\frac{2}{3}$ per cent virus was added to an equal part of serum diluted 2:5 with Tyrodé and to whole serum respectively (columns x and y); and for the final titration, 68 days after inoculation, similar mixtures with 6 per cent virus were made.

As already stated the standard virus fluid yielded one or two discrete papillomas

when diluted to 10^{-4} with Tyrode, but no growths whatever at 10^{-5} on inoculation in standard quantity on a 4 cm. square. It was arbitrarily assumed that at 10^{-4} one infective virus unit was present in the inoculum, and a 6 per cent virus fluid was deemed to contain 600 units. If it was rendered inactive by serum the latter was adjudged to have a titer of 600 or more. The titer on incomplete neutralization was computed from the total number of plus marks recorded for any one inoculum in all the test rabbits receiving this, as compared with the total for the virus-Tyrode control. If, for example, the latter yielded an average of +++ for each of 3 test rabbits, that is to say 9 pluses in all, and the serum-virus mixture yielded + for each animal, or 3 pluses in all, the neutralizing power of the serum was recorded as 6/9 of 600 units or approximately 400. This method of computation was artificial, but it yielded figures that served as a rough index to the differences actually observed. It was not applicable when the control mixtures gave rise to confluent growths, since all concentrations of virus beyond a certain minimum produced these.

The confluent and semiconfluent papillomatous masses resulting from broadcast inoculation of the abdomen appeared between the 14th and 19th day, and by the 33rd day they measured about 6 by 10 cm., and were 5 to 15 mm. high. By the 42nd day they were 10 to 25 mm. high, becoming redundant, and on the day of the last serum test, they were 1 to 3 cm. in height,—enormous, folded, fissured masses with dry tops and fleshy bases. The general health of the animals remained excellent, however, like that of the companion group, save in the case of 3-26, which died of an intercurrent disease on the 37th day.

The tattoo papillomas appeared late, between the 19th and 23rd day in both groups of animals, and at the 68th day they were barely 2 cm. across in the most favorable rabbit (D.R. 3-19). The total skin area involved in papillomatosis and the actual bulk of papillomatous tissue were from the beginning far greater in the group of rabbits with abdominal growths.

Chart 1 depicts the results of this experiment. None of the sera obtained just prior to inoculation had antiviral power. 33 days later this power was pronounced in the case of the animals which had developed confluent papillomatous masses on the abdomen in addition to the small tattoo growths, whereas it was still slight or lacking in the rabbits carrying the latter only. It is conceivable that the difference may have been due in part to the very different amounts of virus introduced into the 2 groups by way of the scarified skin. But a primary immunization induced in this way cannot account for the progressive increase in the antiviral power as the papillomas enlarged. By the 68th day, or long before that, the sera of the group with broad abdominal masses were capable of neutralizing completely a 6 per cent virus extract, 600 or more units of virus, when mixed therewith in

The tattoo papillomas appeared and developed at the same rate in both groups, being to all appearances uninfluenced by the antiviral power of the blood.

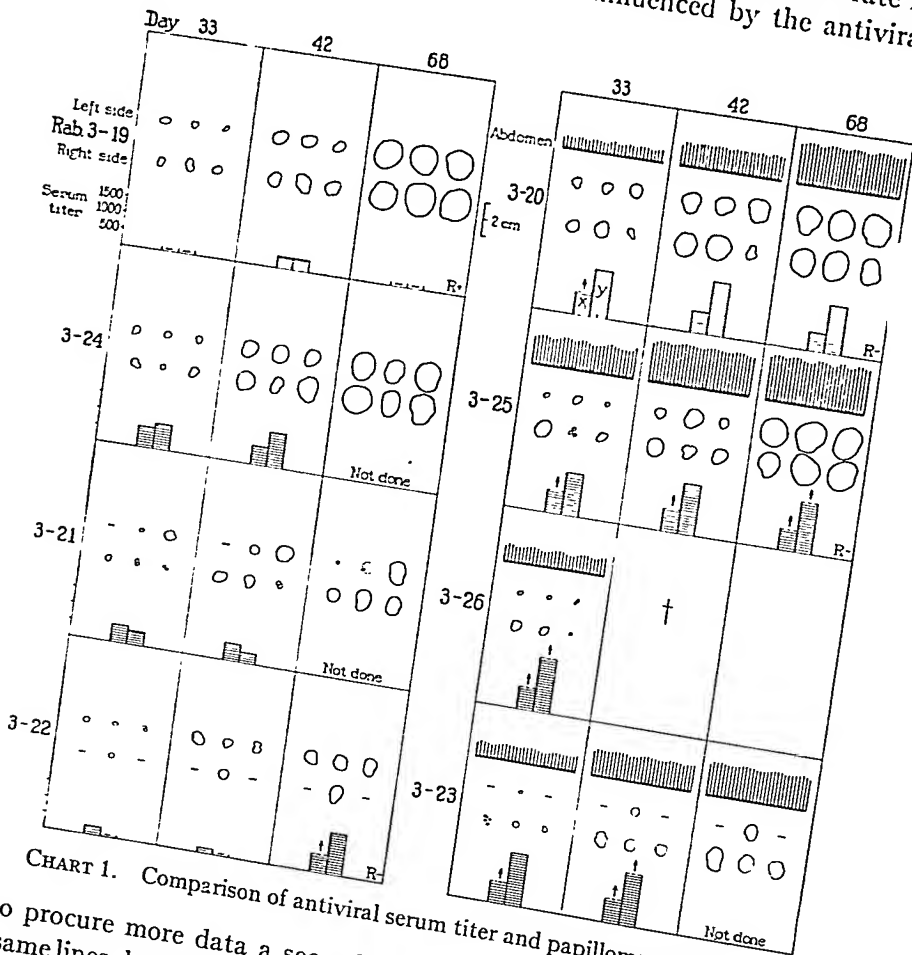


CHART 1. Comparison of antiviral serum titer and papilloma course.

To procure more data a second experiment was carried out along the same lines, but with the addition of intradermal inoculations of the virus. The papillomas resulting from such inoculations are inconspicuous in occurrence, and punctate in origin, developing only where the injection needle has been thrust through the skin. They tend to appear after those from tattoo inoculations and considerably later than

the confluent masses engendered by rubbing in virus broadcast. For all these reasons they seemed especially fitted to disclose whether the neutralizing power of the blood influences the course of events after the cells have become infected with the virus.

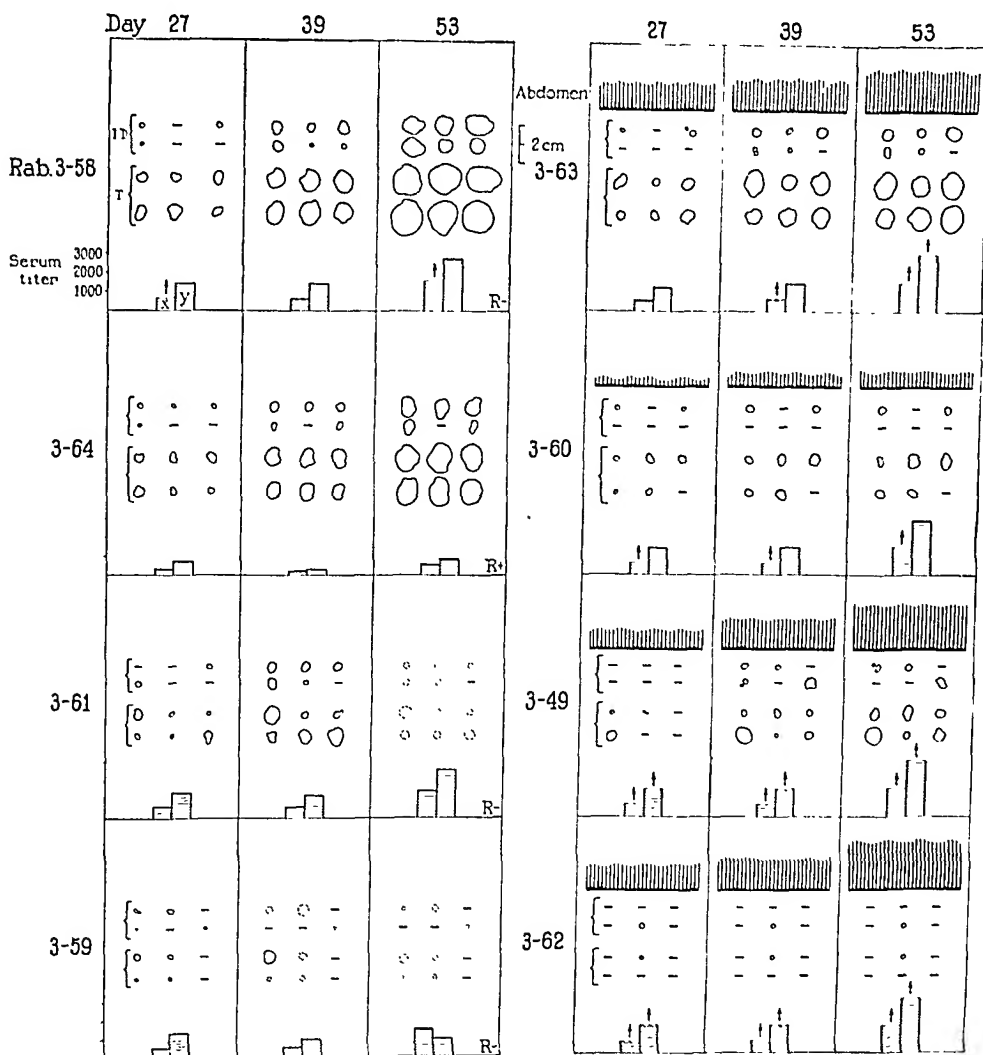


CHART 2. Comparison of antiviral serum titer and papilloma course.

Experiment 2.—In this test the abdominal areas inoculated with virus were notably large, 10 by 12 cm. across. A 5 per cent extract of virus 6-32 was used and both groups of animals received 3 tattoo inoculations in a line on each side, and 3 intradermal inoculations of 0.05 cc. each of fluid immediately above, at a distance of about 4 cm. Serum specimens were procured on the day before the experiment

was begun, and again on the 27th, 39th, and 54th days. On the 64th day 4 of the animals were reinoculated intradermally with 0.3 cc. of a 5 per cent virus extract at 4 sites.

For the preliminary neutralization test the sera were incubated in equal quantity with 5 per cent virus, and 0.5 per cent virus, respectively; for the titrations on the 27th and 39th days 6 per cent virus was mixed in equal parts with whole sera and with sera diluted 2:3 with Tyrode; and for the last titration, on the 54th day, 6 per cent virus was again used, in two mixtures with serum diluted 2:3 and 1:4 with Tyrode.

In 3 rabbits the growth on the abdomen appeared at about the 15th day, occupying the whole field of scarification, and it soon became redundant, folded, and 2 to 3 cm. in thickness. In the fourth animal (3-60), on the other hand, it appeared at about the 20th day, as numerous, discrete and semiconfluent papillomas which slowly attained a height of $2\frac{1}{2}$ mm. The growths from intradermal inoculation 3-62, in which they never developed. The growths between the 27th and 39th day, as became perceptible later, in several instances between the 27th and 39th day, as was to have been expected because of their origin from relatively few cells.

The sera procured just prior to inoculation of the animals were wholly devoid of neutralizing power for the virus, as in the previous experiment. By the 27th day, however, this power was in most instances considerable (Chart 2), and thereafter it underwent a further great increase in 6 of the 8 individuals. The exigencies of charting have made necessary a reduction to half height of the columns representing serum titer. The actual differences in antiviral power in the 2 groups of animals were nearly as pronounced as in Experiment 1.

The sera of the group in which a large amount of papillomatous tissue developed acquired much the greater antiviral power, though this was not true of every animal. It may be pointed out in this connection that rabbit 3-19 of Experiment 1 carried the largest tattoo papillomas of any of its group, 6 growths each nearly 2 cm. across on the 68th day; yet its serum as tested at this time had no perceptible effect to neutralize the virus (Table I), although previously a slight one had been demonstrable; and the final reinoculation resulted in growths. It follows that the presence of a considerable quantity of papillomatous tissue is compatible with a lack of antiviral power. On the other hand there are animals in which this power becomes outspoken although the papillomas responsible for it are small and may endure but a brief time (rabbits 3-22, Chart 1, and 3-59 and 3-61, Chart 2—

animals all of which proved completely resistant on reinoculation). Shope has reported (1) the presence of partial resistance 6 days after the papillomas had first appeared and 14 days after the initial virus inoculation. He had rubbed virus into one entire side of the animals, after appropriate scarification.¹ In rabbits 3-59 and 3-61 of Chart 2 the papillomas were only a few millimeters across at most on the 27th day, yet the antiviral power was already well established.

The intradermal inoculations of Experiment II did not engender

TABLE I

*Neutralization Tests with the Serum of a Rabbit (D.R. 3-19) Bearing Large Tattoo Papillomas
(68th Day after Inoculation)*

6 per cent virus.....	0.5 cc.	0.5 cc.	0.5 cc.	0.5 cc.
Serum D.R. 3-19.....	0.5 cc.	—	0.2 cc.	—
Tyrode.....	—	0.5 cc.	0.3 cc.	0.5 cc.
Test rabbit				
21 days 1	++-	++-	++	++
2	+++	+++	+++-	+++
3	+++-	+++-	+++	++-
27 days 1	++-	++	++	+++-
2	+++	+++	+++-	+++
3	++++-	++++	++++	++++-
34 days 1	++++-	+++	+++-	++++-
2	++++	++++	++++	++++
3	++++	++++	++++	++++-

The plus marks record the character of the papillomatosis produced by the mixtures (see text) as noted on the days given.

growths regularly, nor did the tattooing for that matter; but they appeared in sufficient number for our purposes. The differences in their number and course seem at first sight to parallel the antiviral differences, both the tattoo and intradermal papillomas being more numerous and becoming somewhat larger in the group with the weaker antiviral power; but when the individual instances are scrutinized, one sees clearly that the phenomena cannot be explained by the influence of this power. In rabbit 3-59 with a notably low serum titer the growths all retrogressed between the 39th and 53d days, whereas in

¹ Personal communication from Dr. Shope.

3-63 with a high titer they progressed, as also in 3-58. In 3-61 retrogression took place, although the serum titer was less than in several other animals in which progressive enlargement occurred. Evidently some undetermined factors exercised a decisive influence in these cases. The possibilities are so various that extensive tests with a large variety of controls will be required to determine whether the antiviral power of the blood has any influence whatever upon the papillomatous proliferation, once this has started. Certainly the presence of such power does not suffice to exclude the appearance and development of punctate growths at sites of intradermal inoculation (rabbits 3-49 and 3-63, Chart 2), much less to prevent a vigorous enlargement of growths already well established. There can be no doubt, on the other hand, that the success of reinoculations with virus is conditioned by the state of the blood. 4 animals of each experiment were reinoculated on the 64th and 68th days, and in one instance only in each case did papillomas result,—in an individual with serum devoid of neutralizing power (Chart 1, 3-19)² and in another in which it was slight (Chart 2, 3-64). Shope found 2 papillomatous animals susceptible to reinfection 76 days after primary inoculation. Large growths had resulted from the latter in one of these instances, yet the reinfection gave rise rapidly to growths that seemed equally vigorous. In the other rabbit but few papillomas appeared after reinoculation, and these late, on the broad area into which active virus had been rubbed.¹

The partially neutralizing sera did not cause the papillomas to appear later than on ordinary dilution with Tyrode, nor to grow more slowly or be different. It was plain that the effect of the serum was to cut down the number of effective virus entities, not to alter their individual pathogenic capabilities.

DISCUSSION

The results of rubbing serial dilutions of the Shope virus into areas of scarified skin resemble in some significant respects those from the

² The serum of rabbit 3-19 was tested again after the lapse of 426 days in all. At this time it bore 8 papillomatous masses, of which 6 had been largely replaced by cancers. Its serum now completely neutralized a 0.5 per cent extract of active virus, when mixed therewith in equal parts, and it almost neutralized a 1 per cent extract, its calculated antiviral titer being 90.

seeding of dilutions of bacteria upon agar plates. The discrete papillomas forming in the one case, like the colonies in the other, are the outcome of cell proliferation from individual centers; and the number of effective virus entities can be appraised by the number of growths engendered, just as can the number of other bacteria by the colonies to which they give rise. The inability of the Shope virus to lie latent in the skin of domestic rabbits adds to the reliability of the results, growths appearing soon after inoculation or not at all.

The rabbit sera that partially neutralized the virus might have been expected to cause a delay in the appearance of the papillomas or to alter their character; but nothing of the sort occurred. Instead there occurred merely a reduction in the number of effective virus entities, as expressed in the number of growths engendered. Some entities had been cancelled, but those which remained pathogenic caused growths differing no whit from papillomas due to diluted virus. In this connection the results of attempts to attenuate the virus of poliomyelitis may be recalled, these having brought about in general merely a reduction in the amount of active virus, not an essential change in its character (5).

The blood of domestic rabbits in which virus-induced papillomas are developing nearly always acquires some power to neutralize the virus *in vitro*, whereas that of normal animals is devoid of effect. In only one normal rabbit amongst many has any neutralizing influence been encountered and then it was but slight (rabbit 1, Table IV of Paper II). The absence of antiviral principles from the blood of normal domestic rabbits speaks against the possibility that the papillomatosis now endemic in western cottontails is consequent on an escape of the virus into this species from domestic rabbits in which it has become symbiotic by reason of long association; and the notably vigorous papillomatosis produced by the virus on inoculation into domestic breeds as well as the unfailing susceptibility of all normal individuals would seem to exclude this possibility finally.

The tests with the sera of rabbits carrying tar papillomas and Brown-Pearce tumors reported in Paper II, demonstrate the specificity of the neutralizing power manifested by the blood of animals carrying papillomas induced by the Shope virus. The latter evidently acts as antigen, increasing in amount as the papilloma enlarges in domestic rabbits, although its presence cannot be demonstrated directly.

The blood of the domestic rabbits that we studied acquired antiviral power within a few weeks after papillomatosis appeared, the amount of this power being in general roughly proportional to the skin area involved in the growth. There were some striking exceptions however. The blood of some hosts with small, transient papillomas became antiviral at an early period (Chart 2, rabbits 3-59 and 3-61), while that of one individual developing relatively large growths proved wholly devoid of demonstrable influence (Chart 1, 3-19). It is difficult to suppose that differences in the response of individual hosts to an antigen can account entirely for these phenomena. A possibility exists that the amount of antigen set free from the papilloma may vary with the local conditions. Some papillomas are fleshy, whereas others are dry almost to their base, their epithelium rapidly keratinizing and eventually desquamating. The layer of proliferating, virus-infected cells is supported by very narrow connective tissue cores having vessels that often become blocked. In consequence of these conditions the opportunities for resorption from the mass may differ largely from individual to individual. The thought suggests itself that there may be viruses whose association with cells is so close that they only very exceptionally come away into the host organism in sufficient quantity to elicit an antiviral response on its part. However this may be, the fact remains that some virus-induced papillomas of considerable size call forth little or no humoral response on the part of the host, while other, very small ones cause the blood to become notably antiviral. Puzzling phenomena of similar sort have been often observed in the serological testing of chickens with tumors due to viruses; and they may be due to like conditions.

Reinoculation with the Shope virus was successful in the case of 2 rabbits whose blood had slight or no antiviral power, but it failed in the case of 6 in which this power was more marked (Charts 1 and 2). In a recent experiment the sera of 5 domestic rabbits, in which virus-induced papillomas had retrogressed one year before, were tested for antiviral power by the inoculation of scarified areas with virus of proven activity. Such power was present in all these cases (and in that of rabbit 3-19 after 426 days, as already mentioned), but the titer was low. Nevertheless, reinoculation with active virus gave a negative result in 4 of the rabbits, while in the fifth, with very little antiviral power, it caused 2 tiny growths which soon retrogressed.

No evidence was obtained that the antiviral state of the blood had any effect upon the virus, once the latter had infected the epidermis, but on the contrary there was much to show that it was devoid of influence. Similar observations have been made in the case of a chicken sarcoma caused by a virus (Chicken Tumor I), and an ability of the proliferating cells to protect this virus has been demonstrated experimentally (6). Such protection is known to occur in the case of other viruses as well (7), and the assumption seems reasonable that it is responsible for the continued growth of the rabbit papillomas in hosts with blood of strong antiviral power.

In some of the inoculated animals the papillomas appeared slowly and soon retrogressed, whereas in other individuals with blood of much stronger neutralizing power for the virus they enlarged steadily (rabbits 3-59 and 3-61 of Chart 2 as compared with rabbits 3-58 and 3-63). Primary differences in the suitability of the cells for the virus (8), differences in soil that is to say, will go far to explain such instances. Some rabbits are primarily more susceptible to the action of the virus than are others of the same breed; and the papillomatous growth is generally most vigorous in those individuals whose skins react most pronouncedly to the stimulation of Scharlach R. Observations to be recorded in a future paper by one of us support the view that the host sometimes reacts against the cells of established growths, with result that these retrogress, a happening which is frequent in the case of transplantable tumors. Such an occurrence would explain the slow appearance and uncertain course of the papillomas that appeared at the sites of tattoo and intradermal inoculation some time after papillomatosis had become established over broad abdominal areas (Chart 2).

SUMMARY

A method has been devised for serological tests with a virus producing rabbit papillomas that become carcinomatous. The discrete character of the growths caused by the virus when suitably diluted fits it notably for quantitative experimentation. It shows no tendency to lie latent in domestic rabbits though it does so on occasion in cottontails, the natural hosts. Sera which partially neutralize it do not alter its character, or attenuate it, but merely cut down the number of its effective entities.

The serum of normal domestic rabbits is ordinarily devoid of neutralizing influence on the virus, but that of animals carrying the papillomas usually exhibits neutralizing power soon after these appear. The rate at which this power increases depends in general upon the amount of papillomatous tissue developing, but exceptions to the rule occur, the presence of fairly large growths being compatible with a lack of such powers in demonstrable amount. Even when the antiviral power is great it has no evident influence on the course of established papillomas, other factors determining whether these enlarge or regress. It acts to prevent successful reinoculation of the animal, however.

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EXPLANATION OF PLATE 5

FIG. 1. Results of rubbing mixtures of virus and sera from rabbits carrying papillomas into scarified areas of skin. On one of the squares no growths have appeared; on several there are discrete papillomas in large or small number; and on yet others the growths are semiconfluent or confluent. The confluent proliferation was due to a control mixture with Tyrode.



Photographed by Joseph B. Haulenbeek

(Kidd *et al.*: Virus causing rabbit papillomas. I)

SEROLOGICAL REACTIONS WITH A VIRUS CAUSING RABBIT PAPILLOMAS WHICH BECOME CANCEROUS

II. TESTS OF THE BLOOD OF ANIMALS CARRYING VARIOUS EPITHELIAL TUMORS

BY JOHN G. KIDD, M.D., J. W. BEARD, M.D., AND PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 6

(Received for publication, April 29, 1936)

In the preceding paper a method has been outlined for titration of the antiviral power acquired by the blood of rabbits carrying papillomas induced with the Shope virus. Tests of the blood of animals carrying other epithelial growths will be reported in the present communication.

The Sera of Rabbits with Cancers Originating in the Virus-Induced Papillomas

Carcinomas develop from many of the papillomas induced with the virus in domestic rabbits (1). It was manifestly useless to test the blood of the first hosts of the cancers since they had all carried the preliminary papillomas* for many weeks, and any neutralizing power encountered could be referred to the influence of these growths. The primary cancers themselves yielded an undesirable transplantation material, since papilloma cells might have persisted amidst the carcinomatous elements, to be introduced with the latter into new hosts. For this reason metastases were employed as transplantation material whenever they were available, though in certain instances autoplasmic growths in the leg muscles were utilized. Six attempts at transplantation have been made thus far, all with squamous cell carcinomas. The rabbits employed were of Dutch belted stock but not of wholly pure breed. Detailed histories of the cancers furnishing the materials have been given in a previous paper (1).



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infected with pyogenic bacteria, abscesses developing where they were put.

Rabbit 5-79 developed discrete, smooth surfaced, football-shaped tumors at the sites of implantation (Fig. 1), which were nearly 5 cm. long when cut into after 160 days. Each contained a single large cyst filled with glairy, yellowish fluid, turbid with cell debris. The lining of the cysts was ragged and pink, and their walls nearly 2 cm. thick, tough and close textured, with occasional small yellow dots near the cyst cavity. Slices were taken and the gaps in the cysts' wall were not closed, though the skin was carefully sutured over them. Next day the animal was bled for serum test (Experiment 1). Sections showed the cysts to be lined with the tissue of a rapidly necrosing, anaplastic squamous cell carcinoma, wholly devoid of papillomatous features (Fig. 2), as was the material of original implantation (Fig. 3). The walls consisted in the main of reactive connective tissue into which the carcinoma had extended but a little way, and there it was keratinizing and dying or dead amidst a scattering of lymphocytes.

The gaps left in the cysts healed without extension of the cancers through them, and they enlarged further, with the animal in excellent condition (Fig. 1). After 50 days more (210th day) they measured $6 \times 4 \times 4$ cm., and $5 \times 3\frac{1}{2} \times 3\frac{1}{2}$ cm. respectively. Again they were cut into and the same state of affairs was found as before, both in the gross and microscopically. The animal was bled for another serum test (Experiment 5). 3 days later it succumbed to bacterial infection. The carcinoma proved to be heavily encapsulated everywhere, and there were no metastases.

D. R. 5-84 developed an irregularly rounded nodule in the right fore leg, which reached a diameter of 1.5 cm. within a month after the implantation, remaining stationary for many later weeks. It was perhaps slightly smaller when the animal was bled for serum test on the 161st day, and thereafter it slowly disappeared.

The nature of the nodule present for a time in rabbit 5-84 was never ascertained. The progressively enlarging tumors of 5-79 were of singular character, each consisting of a thin layer of densely encapsulated, carcinomatous tissue, walling a cyst filled with glairy fluid. The original cancer had formed no such cysts and we have seldom encountered similar ones in association with the malignant growths deriving from the papillomas. The great resistance offered by the new host may have had something to do with their development. The living, cancerous lining of the cyst was only 1 to 2 mm. thick and such of the malignant cells as penetrated into the dense encapsulating connective tissue died after but slight proliferation.

Experiment 1 (Table I).—Neutralization tests were carried out in the usual way with serum specimens procured from 5-79 and 5-84, on the 160th day after im-

The general method was to hash the cancerous tissue, suspend it in a relatively large amount of Tyrode solution, and inject 1 cc. into the upper muscles of each leg. The injection needle was introduced through a slit in the skin, with its lips held wide to exclude all possibility that some epidermal cells might be pushed deep and infected incidentally with virus present in the tumor suspension. On withdrawing the needle some of the inoculum always came in contact with the slit skin, but papillomas never resulted.

1. A suspension of the tissue of a metastasis in an axillary gland from a cancer of rabbit 2-38 was implanted in 3 adult rabbits. None developed growths.

2. The tissue of a leg nodule of 2-39, consequent upon autoimplantation, was injected into the legs of 3 rabbits. None developed growths.

3. The basal portion of a cancer of 2-39 was mixed with material from several large nodules resulting from previous implantation of the same cancer into the leg muscles. The tumor suspension was injected into the legs of 9 rabbits and into the testicles of 3 of these. A nodule developed in a leg of one animal (4-70), and biopsy after 79 days disclosed there a squamous cell carcinoma like the original growth (1). Portions were implanted in the legs of 6 adult and 9 unweaned young rabbits, of which latter only 2 survived 3 months. At the end of this time none of the remaining animals had tumors and the growth in D. R. 4-70 had disappeared.

4. An axillary metastasis from a cancer of 2-53 was utilized, and implantation done into the fore legs of 6 unweaned rabbits, 5 recently weaned, and 6 adults. The 8 young that survived for 126 days were all negative then and were discarded. Of the 5 surviving adults one (5-79) had large growths in both fore legs and another (5-84) a nodule 1 cm. in diameter in one of them which disappeared later. Biopsy of the growths of 5-79 on the 160th day showed them both to be squamous cell carcinomas. Tests were made of the neutralizing power of the sera of these animals (*vide* Experiments 1 and 5). After 210 days, 5-79 was again operated upon and material procured for implantation in 15 half grown animals. To the present (3 months) no tumors have appeared.

5. 10 adult rabbits were implanted in the fore legs with a suspension of an axillary metastasis of a squamous cell cancer of 2-38, and in the hind legs with the tissue from an autoimplant of the same cancer in an upper leg. All were negative when discarded 3½ months later.

6. Tissue from another axillary metastasis of 2-53 was implanted in the legs of 15 rabbits 3 months old and 6 adults. One developed a small nodule, which was incised to hasten its growth, but it retrogressed instead. On incision it had appeared cancerous in the gross.

In only 4 of the 96 rabbits did nodules result from implantation, and in 3 of these retrogression soon ensued. The animals furnishing the cancer materials were not of the same genetic constitution as those implanted; while furthermore some of the tissues employed were

infected with pyogenic bacteria, abscesses developing where they were put.

Rabbit 5-79 developed discrete, smooth surfaced, football-shaped tumors at the sites of implantation (Fig. 1), which were nearly 5 cm. long when cut into after 160 days. Each contained a single large cyst filled with glairy, yellowish fluid, turbid with cell debris. The lining of the cysts was ragged and pink, and their walls nearly 2 cm. thick, tough and close textured, with occasional small yellow dots near the cyst cavity. Slices were taken and the gaps in the cysts' wall were not closed, though the skin was carefully sutured over them. Next day the animal was bled for serum test (Experiment 1). Sections showed the cysts to be lined with the tissue of a rapidly necrosing, anaplastic squamous cell carcinoma, wholly devoid of papillomatous features (Fig. 2), as was the material of original implantation (Fig. 3). The walls consisted in the main of reactive connective tissue into which the carcinoma had extended but a little way, and there it was keratinizing and dying or dead amidst a scattering of lymphocytes.

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Experiment 1 (Table I).—Neutralization tests were carried out in the usual way with serum specimens procured from 5-79 and 5-84, on the 160th day after im-

TABLE I

Neutralization Tests with the Serum of Rabbits Carrying Transplanted Cancers Derived from Virus-Induced Papillomas

Rabbits.	5 per cent virus, 0.5 cc. + 0.2 cc. serum + 0.5 cc. Tyrode						1 per cent virus, 0.5 cc. + 0.5 cc. serum						0.1 per cent virus, 0.5 cc. + 0.5 cc. serum						+ 0.5 cc. Tyrode
	L	S	N 1	N 2	C 1	C 2	L	S	N 1	N 2	C 1	C 2	L	S	N 1	N 2	C 1	C 2	
Test rabbit																			
16 1	0	+-	0	++	+-	0	0		++	++	++	++	0	0					
days 2	0	0	++	++	++	++	0		++	++	++	++	++	0					
3	0	+	++	++	++	++	0		++	++	++	++	++	0					
4	0	++++	?	++	++++	++	0		++	++	++	++	++	0					
20 1																			
days 2																			
3																			
4																			
27 1																			
days 2																			
3																			
4																			
34 1	+-	++	++	++	++	++	0	+	++	++	++	++	0	0	+	+	++	0	0
days 2	++	++	++	++	++	++	+	+	++	++	++	++	++	0	+	+	++	++	+
3	++	++	++	++	++	++	+	+	++	++	++	++	++	0	+	+	++	++	+
4	++	++	++	++	++	++	+	+	++	++	++	++	++	0	+	+	++	++	+

L = with large cancers. S = with 1 small cancer presumably. N = implanted with negative outcome. C = normal control.
 0 = negative. +- = 1 or 2 papillomas. ++ = many discrete papillomas. +++ = semiconfluent, discrete papillomas.
 ++++ = confluent papillomas.

plantation. For comparison specimens were utilized from 2 other animals which had been unsuccessfully implanted with the same material, and from 2 normal rabbits of the same breed and approximate age. All had been kept under identical conditions during the period since transplantation. Mixtures were made of equal parts of the sera with 3 dilutions of a virus fluid prepared by passing a 10 per cent extract of virus material W. R. 1240 through a Berkefeld filter V, and then diluting it to 5, 1, and 0.1 per cent with Tyrode. After 2 hours incubation the materials were rubbed into squares on the skin of 4 normal rabbits. Table I records the consequences. As usual growths appeared first where the most concentrated virus fluid had been inoculated (2). The first notations made when the papillomas were barely or dubiously visible, have been omitted from the table. Dilution of the virus fluid to 0.1 per cent brought it close to the working limit of use, beyond which the influence of chance factors as *e.g.* local variations in skin character, in the depth of scarification, and in reactive inflammation, renders the outcome in terms of papillomatosis unreliable for comparative purposes.

It will be seen from Table I that the serum of the rabbit with large carcinomas had a pronounced neutralizing effect on the virus, while that of the animal with a single small nodule exhibited a slighter yet definite antiviral action. None of the control sera influenced the virus. The results of a second test with the serum of 5-79 procured 50 days later, are given in connection with Experiment 5, to be described further on.

The findings appeared to indicate that the Shope virus was present in the animals that developed growths as result of transplantation of the cancer. But there were at least two other possibilities, namely that the neutralizing power exhibited by the sera lay within the range of the normal, or that it was nonspecific, being called forth by the proliferating epithelium as such. Amongst more than 50 normal rabbits tested at various times only one has yielded a serum with any neutralizing power for the Shope virus; but the effectiveness of this serum (Table V, C 1) fell little if at all short of that from 5-84, the rabbit that had developed a small nodule of problematic character. No normal specimen with a neutralizing power approaching that of the rabbit with large transplantation cancers has been encountered; and as will be seen this power still existed at the time of second test (Table VI).

Tests with the Serum of Rabbits Carrying Tar Papillomas

The papillomas which develop on the tarred skin of rabbits are very like those produced by the Shope virus (3). Tests of the serum of

TABLE II
Neutralization Tests with the Serum of Rabbits Carrying Tar Papillomas

1 per cent virus, 0.5 cc. + 0.5 cc. serum										1/5 per cent virus, 0.5 cc. + 0.5 cc. serum								+ 0.5 cc. Tyrode	
Large				Medium			Very small			Large				Me- dium		None			Very small
4-58*	6-17	6-48	6-45	6-49	6-60	6-54	6-55	6-63	+ 0.5 cc. Tyrode	4-58*	6-47	6-48	6-45	6-49	6-60	6-54	6-55	6-63	+ 0.5 cc. Tyrode
Rabbits..																			
Tar papil- lomas..																			
Test rabbit																			
17 days	0	+	+	+	+	+	+	+	+	0	+	+	0	+	+	+	0	+	0
20 days	+	+	+	+	+	+	+	+	+	0	+	+	+	+	0	+	+	+	+
27 days	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40 days	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Carrying very large papillomas for more than 3 months.

animals with tar tumors seemed desirable, both as bearing on the general problem of tumor etiology and as covering the possibility that the neutralizing power of the serum of rabbit 5-79, with large transplantation cancers, represented a non-specific response to epithelial proliferation as such.

Experiment 2, Table II.—9 gray-brown domestic rabbits that had long been tarred¹ were selected from a large group as providing a pronounced contrast in its effects. All had been kept in a separate room, rigidly isolated from contact with the Shope virus. The ears of the rabbit with the largest papillomas (4-58) had been tarred 3 times a week for 12 months, and growths had been present on them for more than 3 months. The remaining 8 rabbits had been tarred thrice a week for 5½ months. 3 of these carried multiple large papillomas (maximum diameter about 2 cm.) of several weeks duration. A fourth had multiple, smaller papillomas up to 1 cm. across. The remaining 4 animals served as controls, no papillomas having appeared on their ears or only minute ones very recently. Serum specimens from all were mixed in equal part with Berkefeld-filtered 1 and 0.2 per cent extracts, respectively, of virus material W. R. 1240. After incubation for 2 hours the materials were inoculated into 3 brown-gray test rabbits. Table II records the results.

None of the sera from the tarred rabbits possessed any neutralizing power whatever save that from 4-58, which had been tarred for many more months than the others, and had larger papillomas of much longer duration. The effect of its serum was slightly more pronounced than that of the generality of specimens from normal animals.

The tarring was continued, the papillomas grew more numerous and larger, and other animals of the group developed them, some of which were included in a second test. In several of the instances with "large" papillomas the crowded, pedunculated growths filled the hollow of the ears, but in none had cancer manifested itself.

Experiment 3 (Tables III and IV).—The sera were procured 9 weeks after those of the first test. All of the animals had tar papillomas now, though in some they were few, minute, and of but a few weeks duration. These are termed "small" in the tables. Sera from 6 normal brown-gray rabbits were taken as controls. Two mixtures were made, with 1 per cent and 0.2 per cent filtered extracts of virus W. R. 1240 respectively. The number of the test mixtures was so great as to preclude the inoculation of all into the same rabbits and hence 3 groups of 3

¹ Horizontal retort tar of the Oster-Gasfabrik of Amsterdam was employed throughout. It was generously given us by Dr. Karl Landsteiner.

TABLE III
Neutralization Tests with the Serum of Rabbits Carrying Tar Papillomas

		1/5 per cent virus, 0.5 cc. + 0.5 cc. serum																			
Rabbits.....		A 4-58*	6-47	6-48	6-45	6-49	6-56	6-46	B 6-51	Controls						Small				B 6-50	+ 0.5 cc. Tyrode
Tar papillomas...										1	2	3	4	5	6	AB 6-60	A 6-54	6-63	6-58		
Test rabbit																					
18 days																					
1	+	0	+	0	0	0	0	0	0	0	+	+	+	+	0	0	+	0	0	0	+
2	+	0	0	0	0	0	0	0	0	0	0	0	+	0	0	+	0	+	+	0	0
3	+	+	+	+	0	+	+	+	+	0	+	+	+	+	+	0	+	+	+	+	+
21 days																					
1	+	+	+	+	+	+	+	+	+	0	+	0	+	+	+	+	+	+	+	0	+
2	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25 days																					
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A = ascites. B = papillomas of short duration.

* Papillomas of over 5 mm.

A = ascites. B = papillomas of short duration.
* Papillomas of over 5 months duration.

test animals each were employed, one receiving only mixtures with 0.2 per cent virus (Table III) while the other mixtures were apportioned as shown in Table IV. The group first mentioned were of Dutch belted stock, and papillomas appeared slightly later in them than in the others, which were of brown-gray breed.

Three of the tarred rabbits had pronounced ascites due to liver cirrhosis consequent on the tarring. With these exceptions they all appeared to be in good health.

The tests yielded no evidence of serum changes referable to the presence of the tar papillomas. Indeed the specimens from some of the animals carrying them appeared more favorable to the virus than those from some of the controls, though the differences were well within the normal range. Rabbit 4-58, with papillomas now filling both ears, yielded a serum that had none of the neutralizing power evident on previous test. The findings as a whole demonstrate that epidermal proliferation, as such, does not cause the serum to become neutralizing for the Shope virus.

Tests with the Sera of Rabbits Carrying the Brown-Pearce Tumor

In further elucidation of the findings serum specimens were studied from rabbits in which the Brown-Pearce tumor was growing or had retrogressed. This transplantable tumor is a swiftly growing carcinoma derived from an epidermal element, presumably of a hair follicle (4). In a previous paper the fact has been reported that animals in which large Brown-Pearce tumors had recently retrogressed developed papillomatosis on broadcast inoculation of the Shope virus at the same time and to the same extent as did normal controls (5). Nevertheless serum tests seem desirable since inoculation with the Shope virus is sometimes successful in rabbits carrying the papilloma, even when their blood possesses some antiviral power.

Experiment 4.—8 normal gray-brown domestic rabbits, from the same source and of approximately the same size, were inoculated with a heavy suspension of freshly prepared Brown-Pearce tumor in Tyrode. 4 others were kept as controls. The tumor was broken up with sand, pressed through a sterile wire mesh, and suspended in 0.9 per cent NaCl. 4 intradermal injections of 0.2 cc. each were made into the loose skin of the back of the neck of each rabbit, as well as intramuscular injections of 0.3 cc. each into the flexor and extensor muscles of both thighs. Both the inoculated animals and the normal ones were kept isolated and in individual cages. The size and character of the tumors were recorded twice weekly. It was

Neutralization Test

	1 per cent virus, 0.5 cc. + 0.5 cc. serum							
Rabbits	A 4-58*	6-49	6-46	B 6-51	1	2	3	AB
Tar papillomas....	Large				Controls			
Test rabbit								
15 days	1	0	+	0	0	0	0	++
	2	+-	+-	0	+++	+	0	+
	3	++	++	+++	++	+-	+	++
18 days	1	+	++	++	++	++	++	++
	2	++	++	++	++	++	0	+
	3	+++	+++	++++	++	+	++	++
21 days	1	++	+++	++	++	+++	++	++
	2	++	+++	++	++	++	++	++
	3	++++	++++	++++	+++	++	+++	++
Rabbits	6-47	6-48	6-45	6-56	4	5	6	6-6
Tar papillomas....	Large				Controls			
Test rabbit								
15 days	1	0	?	0	0	0	0	0
	2	0	+-	0	+	0	0	+-
	3	+	+	+-	++	+-	0	+
18 days	1	++	++	++	++	++	+	+
	2	++	+	+-	++	+-	++	++
	3	++	++	++	++	++	++	++
21 days	1	++	++	++	++	++	++	++
	2	+++	++	++	++	++	+++	++
	3	++	++	++	++	++	++	+++

A = ascites. B = papillomas of short duration.

* Papillomas of over 5 months duration.

bits Carrying Tar Papillomas

bits Carrying Tar Papillomas					1/5 per cent virus, 0.5 cc. + 0.5 cc. serum							+ 0.5 cc. Tyrode	
+ 0.5 cc. Tyrode		A 4-58*	6-49	6-46	6-51	1	2'	3	AB 6-60	6-58	B 6-50		
Large					Controls			Small					
		0	+-	+-	++	++	++	0	++	++	++	+-	++
		++	+	+	++	++	+	++	++	++	++	+	++
		++	+++	+	+++	+++	+	++	++	++	++	+	++
		+	++	+	+	++	++	+	++	++	++	0	++
		+++	++	++	++	++	++	++	++	++	++	+	++
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thought that implantations in the unfavorable skin sites might be followed by early retrogression which in turn would lead to retrogression of the leg growths. Whether for this reason or another, that was the actual course of events in several cases.

Serum specimens were taken from 5 of the implanted rabbits after 50 days. One of these (N 15) had large tumors at all sites, which continued to grow, causing death on the 63rd day. 2 other animals (N 20 and 22) had tumors which had grown well for the first 4 to 5 weeks, attaining a diameter of 2 cm. to 5 cm. in the legs, but then had dwindled and were much smaller at the time of test. In 2 other individuals (N 16 and 18) the tumors reached a fair size in the first 3 to 4 weeks (2 cm. to 4 cm. in the legs) and then rapidly retrogressed, disappearing completely about a week before the test. This was carried out in the usual way. The serum specimens, together with those of the 4 control rabbits, were mixed in equal parts with 1 and 0.2 per cent filtered extracts of virus W. R. 1240. After incubation for 2 hours, inoculation was done into 3 rabbits in the usual way. The findings are summarized in Table V.

The course taken by the Brown-Pearce tumors is expressed symbolically:

$\bigwedge P$ = growing tumors, death later; $\bigvee R$ = good sized, retrogressing tumors; $\bigwedge R$ = fair sized retrogressing tumors; N = negative now, after the retrogression of fair sized tumors.

From Table V it will be seen that of the 9 sera tested, namely those of 5 hosts of the Brown-Pearce tumor and 4 normal controls, only one had any power to neutralize the virus and this derived from a control. The power was but slight, only transiently evident where the mixtures with 1 per cent virus had been inoculated, but it sufficed for the complete neutralization of 1/5 per cent virus.

In a further experiment (Table VI) the serum of other animals carrying the Brown-Pearce tumor proved devoid of antiviral power.

Tests with the Serum of Rabbits Carrying Brown-Pearce Tumors Deriving from Cells Mixed with Shope Virus

It will be recalled that the serum of the rabbit with large carcinomas, consequent on transplantation of a cancer arising from a virus-induced papilloma, had a distinct neutralizing effect upon the virus. The serum of another rabbit of the same transplantation series with but a single small nodule had a slight antiviral influence. These findings appeared to indicate the presence of the Shope virus in the cancers.

Levaditi (6) and Rivers and Pearce (7) have demonstrated that

wholly extraneous, necrotizing viruses, vaccinia (chicken pox), and Virus III, will flourish after experimental introduction into rat, mouse, chicken, and rabbit tumors, endure long after the host has become immune to reinfection with these viruses, and undergo transfer when the growths are transplanted. Such observations suggest that the Shope virus might ride into the cancers deriving from the papillomas it induces, and persist in them, though no longer in a causative relation. As bearing upon this possibility an experiment was carried out to see whether the virus would persist in association with Brown-Pearce tumor cells exposed to infection with it. Incidentally a second test was made of the neutralizing power of serum specimens procured from one of the 2 rabbits above mentioned which carried transplanted cancers deriving from virus-induced papillomas.

Experiment 5.—A suspension of Brown-Pearce tumor cells prepared as in Experiment 4 was mixed in equal volume with 5 per cent filtered Shope virus fluid (W. R. 1240), and with Tyrode solution respectively. The two mixtures were allowed to stand at room temperature for 1 hour, and then 0.5 cc. of each was inoculated into the fore and hind legs of 3 normal, brown-gray rabbits. The injections were made into the upper leg muscles through skin slits held wide, those of the same inoculum on the same side of the animal. On withdrawing the needle the inoculation fluids were purposely allowed to come in contact with the skin wounds; and wherever they had contained virus, a skin papilloma developed later. None appeared where the mixture with Tyrode had been introduced.

One animal was killed on the 18th day. Brown-Pearce tumors had developed at all of the implantation sites, those derived from the cells exposed to virus being much the smaller. The second rabbit was killed on the 31st day. It too had tumors at all of the inoculation sites. The combined weight of those from the cells exposed to virus was 4.8 gm., whereas the tumors from the Tyrode-soaked cells weighed 18.0 gm. The last animal was killed on the 35th day. Again the tumors engendered by the material containing virus were much the smaller, weighing 17 gm. as compared with 54 gm. for the controls. Metastases were present in the axillary and groin lymph nodes on both sides. All of the growths had the same histological character.

The tumors from the first animal were utilized for further transplantation. Those from the two sides were suspended separately in Tyrode and inoculated with the same technique as before into the legs of 4 normal brown-gray rabbits. In addition, 0.2 cc. of the injection material presumably containing virus was inoculated intradermally at 4 sites in the loose skin over the nape of the neck.

Tumors appeared in all of the new hosts. By the 46th day, when serum tests were made, they had reached a diameter of 2 to 4 cm. in one animal (N 57). In another (N 59) they had grown as large but were now retrogressing, while in the

TABLE VI

Neutralization Tests with the Serum of (a) a Rabbit with Transplanted Cancers Derived from Papillomas, (b) Rabbits with Brown-Pearce Tumors Deriving from Cell Material Exposed to the Papilloma Virus

5 per cent virus 0.5 cc. +				1 per cent virus 0.5 cc. +				1/5 per cent virus 0.5 cc. +			
Serum		0.5 cc.	0.2 cc. 0.3 cc.	0.5 cc.	0.5 cc.	0.5 cc.	0.5 cc.	0.5 cc. serum		0.5 cc. serum	
Tyrode.		—	—	—	—	—	—	—		—	
(a)											
(b)											
(c)											
(d)											
(e)											
(f)											
(g)											
(h)											
(i)											
(j)											
(k)											
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remaining 2 hosts (N 56 and 58) they had attained a diameter of 2 to 4 cm. at an early period but then had rapidly dwindled, disappearing a few days before the sera were procured. The size of the tumors produced by the 2 inocula did not differ noticeably, and no papillomas appeared where the skin had been slit for inoculation.

Rabbits 5-79 and 5-83, to which a cancer deriving from a papilloma had been transplanted, were also bled for serum. The growths of 5-79 had enlarged since the serum test 50 days before (Table I, L), but no tumors had ever appeared in 5-83. It had been amongst the controls of the previous test.

Mixtures were made of each of the sera, and of those of 2 normal, brown-gray controls, with an equal part of 1 per cent and 1/5 per cent filtered virus fluid respectively, and with a 1/5 per cent fluid made up from the 1 per cent material (strain 1240). After 2 hours incubation all were inoculated into the skin of 3 normal brown-gray rabbits, one of which died of intercurrent causes a few days later. The growths appearing in the others were recorded as usual (Table VI).

In this experiment the tumors resulting from the implantation of Brown-Pearce cell material mixed with Tyrode and with virus respectively were transplanted to a second set of hosts, and the sera of the latter were tested for antiviral power in due course. They proved wholly devoid of it.

The initial activity of the virus was sufficiently shown by the development of papillomas where the skin was injured during implantation of the mixture containing it together with Brown-Pearce tumor cells; and the growths deriving from this mixture were smaller than those produced by the implantation of cells unexposed to virus, although no histological differences were discernible. On further transplantation no papillomas arose at the sites of skin injury,² and the tumors did not differ from the controls in size or aspect. The findings corroborated those of Experiment 4, in which the serum of animals carrying the Brown-Pearce tumor, or in which it had recently retrogressed, was found to have no neutralizing effect for the papilloma virus, and they go further, proving that the virus does not persist sufficiently long in Brown-Pearce tumors to be carried over into new hosts together with the cells of these growths. The findings differed essentially from those in the case of the rabbit with large transplanted carcinomas derived

² This failure of papillomas to develop cannot be considered significant, since virus of the strain employed has never been recovered in active form from the papillomas engendered therewith in domestic rabbits.

from a virus-induced papilloma. The serum of this animal once again gave evidence that the Shope virus had been transferred together with the tumor material.

DISCUSSION

The cancers deriving from the papillomas have proved difficult to transplant to other animals of impure breed,—in which respect they resemble the tar cancers,—and in only one case has the implanted material given rise to large, indubitable carcinomas. In this instance the possibility that papilloma cells had been carried over into the new host was excluded by the utilization of a metastasis for the transfer. The serum of this host proved neutralizing for the papilloma virus on two occasions separated by an interval of more than 7 weeks. Its titer was low, however, as measured by the standard described in Paper I; it neutralized not more than 20 virus units when mixed with virus suspension in equal amount, whereas the blood of some rabbits carrying papillomas rendered inactive 3000 units or more. But it may be recalled that the serum of D. R. 3-19 (Chart 1, Paper I) had no neutralizing power whatever, at a time when it supported much more papillomatous tissue than the cancer animal did of malignant tissue in the curious cystic carcinomas. The blood of a second rabbit of the same group as the latter, with a small nodule of problematic character, resulting from transplantation, exhibited some neutralizing power.

Even in the case of the rabbit with large cancers the findings mean no more than that the Shope virus underwent transfer with the cancer, an expected finding, since wholly alien, necrotizing viruses often thrive in tumors into which they are experimentally introduced, and they can be transferred therewith. In view of this fact it is remarkable that the papilloma virus did not persist in Brown-Pearce tumors exposed to infection with it, for these growths are certainly of epithelial, and presumably of epidermal, nature. The virus, if active, could scarcely have been outgrown and left behind, for it attends the growth of the papilloma which proliferates with great rapidity. The remarkable specificity of the virus, which will not "take" on the gums and tongue of rabbits, though highly effective on the neighboring epidermis, may explain its disappearance.

The findings with serum specimens procured from animals carrying tar papillomas or the Brown-Pearce tumor speak decisively against the possibility that these growths are caused by viruses antigenically related to the one causing papillomas. Yet this does not exclude a virus causation for them, since the sera of fowls with Chicken Tumor I and Fujinami sarcoma respectively, though possessed of neutralizing power for the virus causing the growth carried by the host, have no cross-neutralizing effect whatever (8).

SUMMARY

The serum of a rabbit with large cancers resulting from the transplantation of a squamous cell carcinoma that had arisen from a virus-induced papilloma, possessed the power to neutralize the virus, and so too in less degree did that of an animal of the same transplantation series in which a small nodule had developed. The sera of rabbits carrying tar papillomas or the Brown-Pearce carcinoma proved wholly devoid of effect on the virus. The implantation of Brown-Pearce tumor material mixed with virus did not lead to an enduring establishment of the latter in the resulting growths, nor to any immediate changes in their morphological character.

The significance of the facts is discussed.

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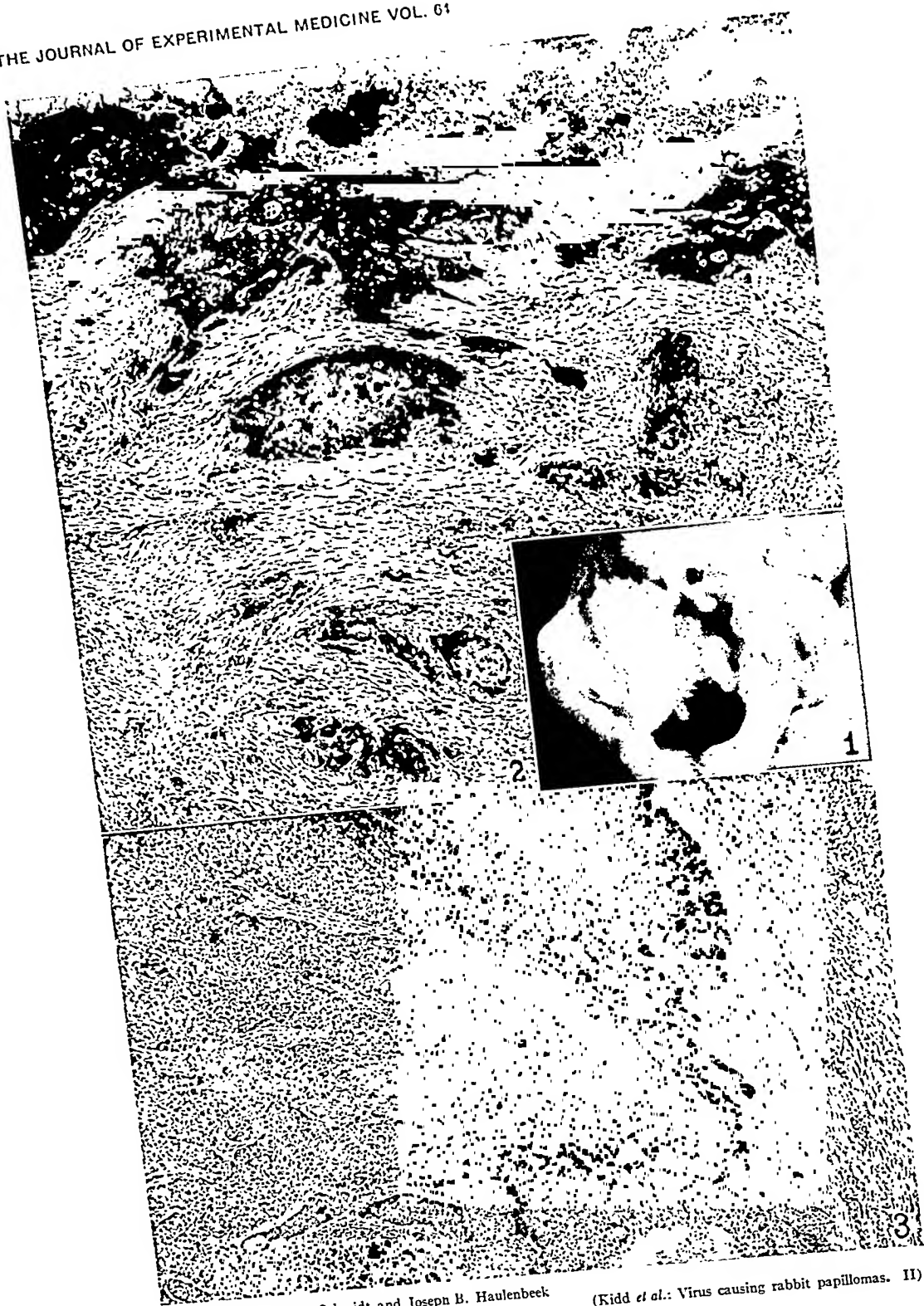
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EXPLANATION OF PLATE 6

FIG. 1. Cancers consequent on transplantation to the fore legs of D. R. 5-79: 190th day. $\times \frac{1}{3}$.

FIG. 2. Inner portion of the wall of one of the cystic growths shown in Fig. 1. Methylene blue and eosin. $\times 30$.

FIG. 3. Growing edge of the axillary metastasis utilized for transplantation to D. R. 5-79. Methylene blue and eosin. $\times 30$.



Photographed by Louis Schmidt and Joseph B. Haulenbeck

(Kidd *et al.*: Virus causing rabbit papillomas. II)

CHANGES IN THE BONE MARROW AND BLOOD CELLS OF DEVELOPING RABBITS

By F. R. SABIN, M.D., F. R. MILLER, M.D., K. C. SMITHBURN, M.D.,
R. M. THOMAS, M.D., AND L. E. HUMMEL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 7 TO 9

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We have studied the cells of the blood and bone marrow of young rabbits, starting with late fetal stages and following the changes that take place at birth and during the first 6 months of life. By that time the rabbit is mature and the different strains of cells in the blood have reached the proportions characteristic of the adult. The changing pattern of the blood cells finds its explanation mainly in changes in the bone marrow.

As is known, the tissues of the so called marrow cavity are not at once associated with the formation of blood cells. Rather the first function of this organ is in relation to the development of the bone. In the rabbit the changes in the original cartilage begin in the center of the shaft of the long bones during the 17th and 18th days of gestation (1). The primary vascular pattern is of large sinusoids which, beginning in the center of the shaft, soon extend to form loops between the lines of the cartilage cells at each epiphyseal line. Between the sinusoids there is a delicate reticulum containing sparsely scattered connective tissue cells embedded in a gelatinous matrix. There are no fat cells. Along the edges of the developing bone and the regressing cartilage there are osteoblasts or multinucleated osteoclasts, the latter formed, as Arey (2, 3) has shown, by the fusion of osteoblasts. The study of bone marrow as an hematopoietic organ is concerned with the method by which this simple structure becomes changed to provide for the development of three different strains of cells, namely, red blood cells, granulocytes, and megalokaryocytes.

TABLE I			
Blood Cells of 115 Rabbits for the First 6 Months of Life			
	Granulocytes	lytes	tes

[illegible]

4th wk.	23	23 R.B.C. 19 Hb 23 W.B.C.	2,300	5,124,000	70	0.70	4,267	1,508	523	32	1,521	670	2	—	—	—	8	—	—
2nd mo.	35	94 R.B.C. 81 Hb 95 W.B.C.	9,500	5,434,000	71	0.55	4,927	2,258	405	39	1,411	792	4	—	11	—	3	1	—
3rd mo.	31	85 R.B.C. 75 Hb 85 W.B.C.	8,500	5,501,000	66	0.58	5,961	2,515	379	46	2,173	826	1	—	17	—	3	1	—
4th mo.	21	51 R.B.C. 41 Hb 51 W.B.C.	5,100	5,639,000	64	0.56	6,404	2,690	469	78	2,513	626	2	—	11	—	9	1	—
5th mo.	24	70 R.B.C. 68 Hb 70 W.B.C.	12,400	5,602,000	73	0.65	7,924	3,305	448	167	3,266	721	6	—	3	—	3	1	—
6th mo.	29	100	22,600	5,751,000	79	0.68	9,829	4,152	500	137	4,069	954	8	—	1	—	4	2	—

Materials and Methods

132 rabbits were used in the experiment. They were bred at the Institute and all of each litter were used. Blood counts were made on 115 rabbits and counts of the marrow cells on 49. 32 animals had studies of both blood and marrow cells. Since some of each litter were killed from time to time for the studies of the bone marrow, other rabbits were added for the blood counts of the 5th and 6th months. In the earlier group, the animals were of several different breeds, including a few New Zealand Reds; the animals added for the 5th and 6th months were all New Zealand Reds.

For the fetal stages and the first 2 weeks of life the blood was obtained by heart puncture; after that from an ear vein. The platelets are high in early life and the blood clots with extreme rapidity. It is thus necessary to put the blood from the syringe into a paraffined watch glass and have different people take the blood for the several preparations immediately and simultaneously.

The preparations of the bone marrow were made from three bones, the femur, the tibia, and the humerus. The supravital technique (4-7) was used entirely for the counts of blood cells. This technique has a great advantage in the study of the blood cells of animals since it allows a better discrimination of the monocyte. The granulation of this cell, to be seen in fixed films of human blood, is lacking in the corresponding cell in rabbits, probably correlated with the fact that the monocyte in the blood of rabbits is oxidase-negative. The lack of this granulation makes difficult its discrimination from the lymphocyte in fixed films of rabbits' blood. In the supravital technique the differentiation of the monocyte is made by other characteristics,—the vacuoles, the mitochondria, the surface film, and the type of motility.

In making the differential counts of the blood cells, 100 cells were counted from each animal through the 4th month, as shown in Table I. For the 5th month, 10 of the animals and 22 of the counts were from the original series and hence of 100 cells each; 14 of the rabbits were from the new series, with 48 counts of 200 or 400 cells each, making a total of 12,400 cells counted. For the 6th month only 7 animals were left from the first series, making 700 cells counted from this group. The other animals, 93 in all, had counts of 200, 400, 500, or in two instances of 1,000 cells, making a total of 22,600 cells. In the entire series for all ages, 73,100 cells were counted. In general our present procedure in making blood counts is to count 100 cells from each of 2 drops of blood and use the average in case the 2 counts correlate. If they do not, to count from 400 to 1,000 cells from fresh preparations.

For the counts of marrow cells, it is our opinion that the supravital technique is also preferable, since the method allows more accurate differentiation and the alteration of cells is minimal. All the counts of the marrow cells recorded in the table and on the graphs were made by this method. In 15 instances duplicate counts of the marrow cells were made from fixed films. The correlation was good in 7 and poor in 8 counts, and it is our opinion that the supravital technique is the

better standard. For the fetal marrow and for that during the first 2 weeks of life, the preparations of the marrow can be made either by sucking the marrow a short distance into a capillary pipette and transferring to the slide, or by squeezing the entire marrow out of the bone after carefully removing muscle, tendon, and periosteum from its surface. In the early stages the marrow is so fluid that it spreads readily with the weight of the coverslip; in later stages, when fat has appeared, a tiny bit of the marrow, placed on a slide, can be spread by slow and gentle pressure on the coverslip.

The counts of marrow cells cannot be as accurate as blood counts since there must be a choice of fields. Only zones in which the cells are in a single layer and slightly separated from each other can be counted; also they must be well stained. For these reasons it is not possible to count systematically across a film and back, as with films of blood. These factors make it advisable to count more cells than for blood and as a result of our studies we think that 5,000 cells should be the minimum for each animal.

The cells of the marrow can also be counted from sections if they meet certain standards. The fixative, as Maximow (1) showed, should be Zenker-formol; the formol should be free from acid. The absence of acid prevents the solution of hemoglobin and of certain granulations. The sections must then be so thin, not more than 4μ , that the cells are all in a single layer, and the staining, Giemsa preferred, such as to give maximum discrimination.

RESULTS

Blood Counts

The pattern of the blood cells of these young rabbits is shown on Chart 1,¹ from data which are given in Table I. In making the chart, the intervals of time for the fetal period are spaced arbitrarily. Rabbits are born sometime between the 28th and 32nd day after mating. As is shown on Chart 1, the number of red cells in the rabbit's blood is still low in the late fetal days, with a tendency to rise on the day of birth. In the 7 animals of a litter counted on the 28th fetal day, the range was wide, varying from 2,210,000 to 3,290,000 cells, and there was a variation in hemoglobin of 13 points. On the 29th fetal day, the range was less, namely, from 3,205,000 to 3,865,000 cells, with the hemoglobin varying 12 points. All but one in this group were from the same litter and this one had a count near the average, namely, 3,490,000 cells. The drop in red cells, shown on the 30th day of gestation, has probably little significance, since this was a single count taken from one of a litter. The rise on the day of birth was, however, significant, only one animal of the 22 counted falling below 3,000,000 cells. The range was from 2,760,000 to 5,210,000 cells and 16 of the rabbits had counts between 3,400,000 and 4,400,000 red cells.

¹The figures represented on Charts 1, 2, and 3 are recorded as logarithms so that the relativity of each change is better expressed.

CHANGES IN BONE MARROW AND BLOOD CELLS

During the rest of the 1st week of life, there was a slight fall in erythrocytes, amounting to 250,000 cells. Of the 33 animals counted, 21 had counts under 4,016,000, the average on the day of birth, but in the group there were 4 animals which had 3 counts during this period, namely, on the 2nd, the 4th, and the 6th days of life. Of these 4, 2 showed a steady rise in the red cells, while one had a fall of 500,000 red cells on the 2nd count, with recovery on the 3rd, and the 4th had a fall of 800,000 cells on the 3rd count. The loss in red cells in these 2 rabbits may well have been due to the fact that for the heart punctures, 3 in number,

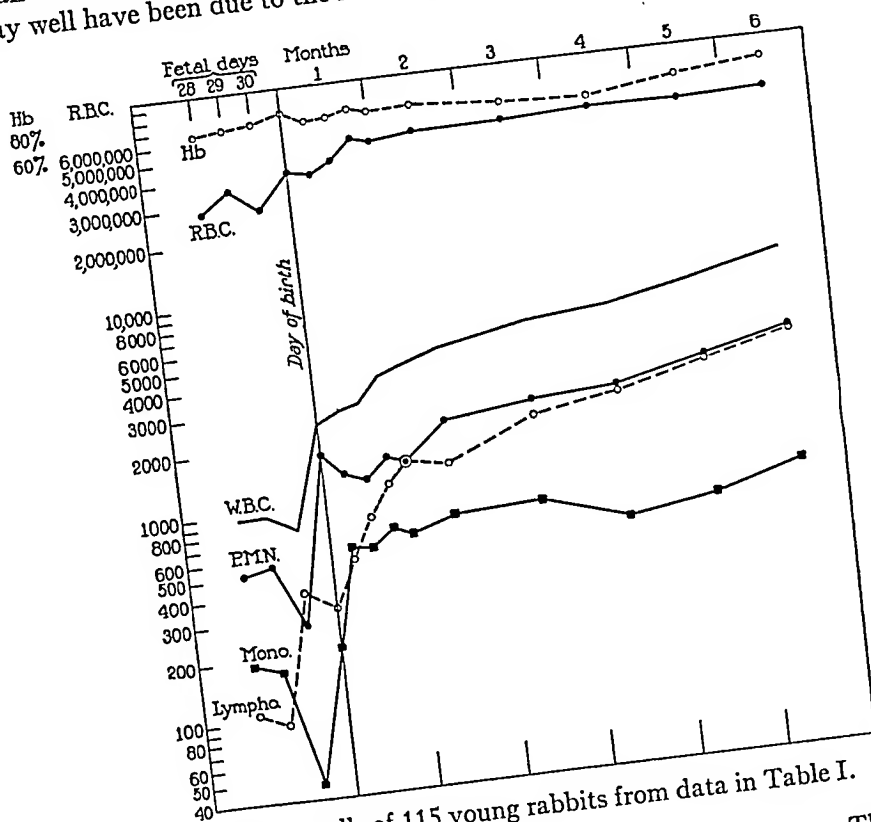


CHART 1. Blood cells of 115 young rabbits from data in Table I.

more blood must be taken than for the later counts from the veins. These two records, therefore, cannot be regarded as a physiological fall in red cells. But if the counts of these 2 animals are omitted from the series, the average of the other 27 counts still shows a fall in red cells, namely, to 3,744,000. The loss, 250,000 cells, is slight, but at least the data establish the fact that the rabbit is unable to produce enough mature red cells to cause a rise in their number in the blood during the 1st week of life.

During the 2nd and 3rd weeks, on the other hand, there was a remarkable rise in red blood cells to a mean level in the 3rd week of life of 5,399,000 cells, which is the established mean of red cells of the adult rabbit. Adequate figures on this

subject have been given by Pearce and Casey (8). For reference, we give Table III.

It will be noticed on Chart 1 that there was a slight drop in red cells in the 4th week, which may indicate a slight reaction after the remarkable activity of the bone marrow toward red cells during the 2nd and the 3rd weeks. The estimation of hemoglobin was made by the colorimetric method with the Newcomer standard. It is known that the colorimetric method has a wide range of error. To limit this error as much as the method permits, all of the readings in the first series were made by one person and the same was true of the second series introduced in the 5th and 6th months. The line of the percentage of the hemoglobin on Chart 1 is, notwithstanding, a remarkably straight line. In column 7 of Table I is given the color index which is shown graphically on Chart 1 by the spread between the lines of the number of red cells and the percentage of hemoglobin. The proportion of hemoglobin per red cell is high in fetal blood, as has recently been shown by Wintrobe and Shumacker (9) and by Kunde *et al.* (10). Chart 1 shows that this condition persists for a time after birth and that the amount of hemoglobin per red cell only gradually approaches the standard of the adult rabbit, which is reached in the 3rd and 4th months of life. The rise in color index for the 5th and 6th months may well be due to a predominance of one breed of rabbit.

The lower half of Chart 1 shows the total numbers of the white blood cell, together with the proportions of three of the strains, namely, the neutrophilic, that is, pseudoeosinophilic,² leucocytes, the lymphocytes, and the monocytes. The differential counts are recorded in Table I in terms of total numbers rather than percentages. It will be noted that in contrast to the red cells, the white cells of the blood increase slowly and do not reach the numbers characteristic of the adult animal until the 5th and 6th months. For comparison, our data on the blood cells of the adult rabbit are given in Table IV.

As shown on Chart 1 and Table I, the white blood cells were low in the circulation during the late fetal stages, the average being about 900 cells per c.mm. In the series of 16 counts, the range is from 700 to 1,425 cells; only one count was above 1,175 and 10 of the 16 counts were below 1,000 cells. As with the red cells, there was a sharp rise on the day of birth from the average of 900 to 2,525 cells. On the day of birth there was a wide variation in the limits of the counts, but only one was below 1,200 cells. This was a single count of 300 cells, balanced by 2 high counts of 5,000 and 7,000 cells. Most of the counts, however, fell between the limits of 1,200 and 2,700 cells, with 5 counts of 2,100.

As seen on Chart 1, the rise of the white cells that took place at birth was almost wholly due to neutrophilic leucocytes. This tendency was, however, not sustained during the 1st month, and it was not until the 2nd month that there was initiated the progressive increase which carried them to their full quota in the 5th and 6th months.

²The term neutrophilic is used throughout for the pseudoeosinophilic granulations of rabbit's blood.

Accessory

TABLE II

Cells of the Bone Marrow of 49 Rabbits

		Myeloid										Accessory								
Animal No.	Age	No. of cells counted	Erythroid				Total	Neutrophilic leucocytes	Basophilic leucocytes	Eosinophilic leucocytes	Myelocytes C	Myelocytes B	Myelocytes A	Myeloblasts	Total	Primitive cells	Megakaryocytes	Plasmacytes	Osteoclasts	Unclassified
			Normoblasts	Late erythroblasts	Early erythroblasts	Megaloblasts														
R 1170 A*	Late fetal	750	2.80	2.13	1.33	—	6.26	0.66	—	—	0.40	12.66	13.86	55.49	83.07	8.13	—	—	2.13	—
4311	29th fetal day	5,000	22.74	40.56	10.60	0.18	74.08	1.84	0.64	0.08	1.74	4.68	3.02	1.74	13.74	9.88	0.06	1.98	0.26	—
4312																				
4313																				
4314																				
4302	30th fetal day	5,000	22.0	41.06	8.1	0.56	71.72	5.82	0.16	0.12	4.42	5.16	1.22	—	16.90	11.0	—	0.16	0.02	0.20
4303																				
4304																				
4305																				
4306																				
1658	Day of birth	3,550	21.07	14.56	14.00	1.27	50.90	4.90	0.39	0.14	6.02	6.00	5.07	13.43	35.95	11.35	0.47	0.92	—	0.36
1588																				
1627																				
1640																				
1779	2nd day	4,219	9.05	12.51	6.94	0.87	29.37	10.76	0.85	0.14	7.70	10.64	6.99	16.09	53.17	15.71	0.11	0.63	0.26	0.71
1780																				
1630																				
1424																				
4374	3rd day	5,000	28.32	17.30	3.54	0.01	49.17	28.34	1.70	0.42	4.32	3.76	3.52	0.40	42.46	7.02	0.08	0.90	0.04	0.32
4375																				
1458	4th day	6,000	39.60	34.51	4.58	0.63	79.32	6.63	0.80	0.23	2.58	1.91	1.66	0.23	14.01	5.4	0.25	0.65	0.11	0.18
4376																				

CHANGES IN BONE MARROW AND

1784	5th day	20,000	23.50	33.62	6.08	0.16	63.36	11.16	1.24	0.44	3.84	2.98	4.52	2.58	26.76	8.75	0.05	0.89	0.06	0.08
4377	6th day	5,000	17.48	38.22	3.38	—	59.08	15.94	1.84	0.52	3.30	4.52	6.24	3.14	35.50	3.34	0.26	1.54	0.06	0.22
4396	7th day	2,660	51.57	15.52	13.60	2.93	83.62	2.44	0.26	—	10.37	1.80	0.78	—	15.65	0.30	0.30	0.03	—	0.03
4397	2nd wk.	9,991	35.12	36.21	2.28	0.31	73.92	11.01	0.31	0.19	5.01	2.57	2.27	1.26	22.62	2.85	0.11	0.41	0.01	0.05
4398	3rd wk.	1,894	36.85	9.13	9.13	0.58	55.69	14.46	0.21	0.03	17.84	5.38	0.81	0.10	38.88	4.75	0.26	0.36	—	—
4399	4th wk.	3,193	28.78	11.65	5.48	0.50	46.41	22.61	1.00	0.43	14.62	8.76	1.25	1.91	50.61	1.62	0.46	0.40	—	0.43
1286	2nd mo.	16,559	32.85	24.40	2.95	0.03	60.23	22.56	1.26	0.67	7.19	2.86	1.97	0.27	36.78	2.49	0.19	0.22	—	0.03
1470	3rd mo.	2,000	40.80	7.60	5.20	1.30	54.90	3.70	1.05	2.65	32.10	3.15	1.40	0.30	44.35	0.30	0.30	0.15	—	—
1466	4th mo.	2,000	25.05	8.05	4.25	0.75	38.10	13.45	3.00	0.85	32.85	2.60	1.35	0.70	51.80	6.15	0.80	0.15	—	—
1466	5th mo.	6,235	29.60	21.18	0.94	0.08	51.80	31.54	1.04	1.01	7.58	2.58	2.43	0.09	46.30	1.57	0.20	0.10	—	—

* These are serial numbers of the department covering a term of years.
† The date of mating was not known and hence this record is not shown on Charts 2 and 3.

CHANGES IN BONE MARROW AND BLOOD CELLS

Quite different from this are the curves for lymphocytes and monocytes. The lymphocytes showed a progressive rise in total numbers from the exceedingly low levels of the late fetal stages to their full quota in the 5th and 6th months of life. This rise was more rapid during the 1st month than thereafter. On the other hand, the monocytes rose rapidly during the 1st week of life to their final quota, which was maintained for the rest of the 5 months. Thus the monocyte is the first cell in the blood stream to reach its normal quota.

TABLE III
Red Blood Cells in Rabbit

	Mean	Mode	Probable error	Standard deviation	Coefficient variation per cent
Pearce and Casey (data on 174 normal adult rabbits)	5,198,000 \pm 12,700	5,215,000	432,748	628,250	12.09
Data from this laboratory on 62 normal adult rabbits*	5,366,000 \pm 96,406	5,400,000	440,000	660,000	12.30

* For these data the blood cells of the 62 rabbits were counted 456 times; the mean for each rabbit was obtained and used in calculating the frequency distribution from which the probable error and standard deviation were determined.

TABLE IV
*White Blood Cells in the Rabbit (Data on 62 Animals)**

	Mean	Mode	Probable error	Standard deviation	Coefficient variation per cent
White blood cells.....	8,866	3,000	1,158	1,720	31
Neutrophilic leucocytes.....	3,727 (42.03%)	500	342	508	47
Basophilic ".....	715 (8.06%)	—	—	—	—
Eosinophilic ".....	110 (1.24%)	—	952	1,422	37
Lymphocytes.....	3,595 (40.54%)	3,750	356	528	49
Monocytes.....	719 (8.10%)	500	—	—	—

* See also the figures of Pearce and Casey (8).

Table I also gives the data for basophiles and eosinophiles. The basophiles rose steadily for the 1st month and then remained at a nearly uniform level for the next 5 months, but they did not reach, during this period, the level previously determined as characteristic of the adult rabbit. Our figures are 715 basophilic leucocytes per c.mm. (Table IV) and those of Pearce and Casey, 950 (8). The eosinophilic leucocytes, on the other hand, remained below 100 cells for the first 4 months of life but reached their adult level, namely, 110 cells per c.mm., during the 5th and 6th months. Pearce and Casey (8) give a higher figure, namely, 200 leucocytes per c.mm.

The occurrence of certain accessory cells in the blood stream is indicated in Table I. The most interesting points are the presence of myelocytes in the circu-

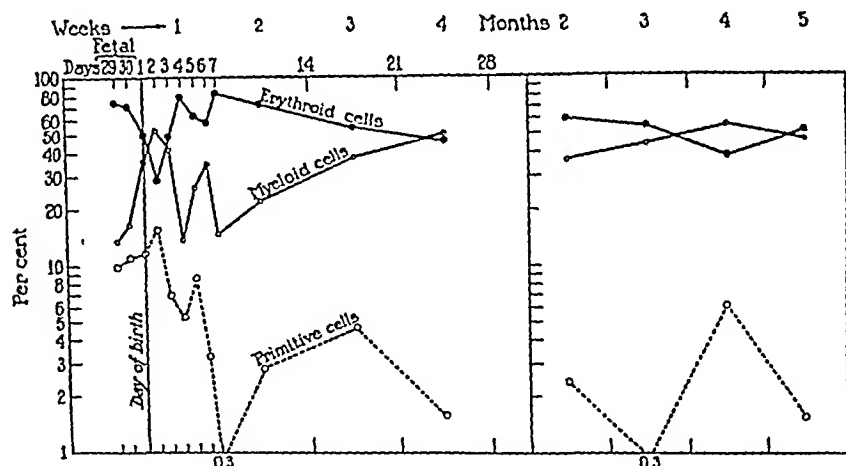


CHART 2. Three major strains of cells in the bone marrow of 49 young rabbits from data in Table II.

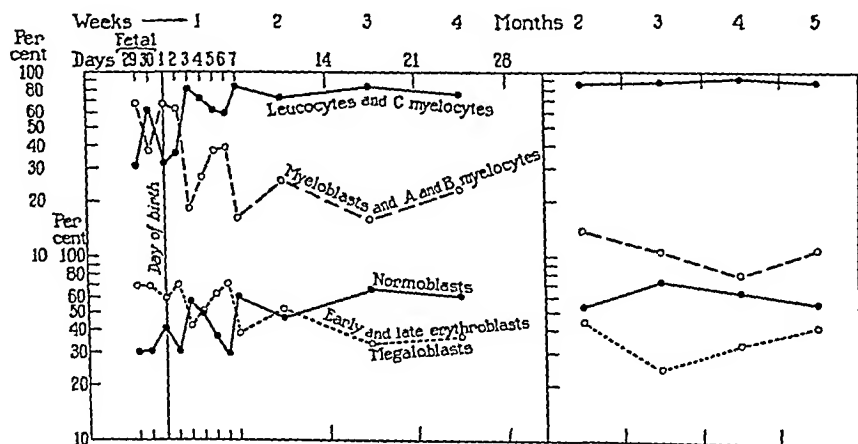


CHART 3. Relative proportion of mature to immature cells in both erythroid and myeloid strains of the bone marrow of 49 young rabbits from data in Table II.

lation in the fetal stages and on the day of birth. The blood of one of the animals, R 1292, counted on the day of birth, had a large number of megalokaryocytes, 8 per cent or a total of 168 per c.mm.

CHANGES IN BONE MARROW AND BLOOD CELLS

Differential Counts of the Cells of the Bone Marrow

The records of the studies of the cells of the bone marrow are shown on Charts 2 and 3, from data given in Table II. Since there are no total counts of the cells of the marrow, the curves are plotted in percentages. The figures for Table II were obtained by taking the percentages of the total number of cells counted in all of the animals of a given age. Chart 2 gives the proportion of erythroid, myeloid, and primitive cells in the marrow, while in Chart 3 the data are analyzed to show the proportions of mature to immature cells in the erythroid and myeloid strains. For Chart 3 these two strains were considered as 100 per cent each and the percentage computed on that basis.

From the studies of the cells of the blood of the rabbit, it has been clear that the erythroid cells reach their final level much earlier than the myeloid types. This fact is reflected, or rather explained by the condition of the bone marrow, as shown in Chart 2. If the total numbers of cells in the marrow are considered, it is probable that there is a constant increase in both strains of cells but the disproportionate increase in erythroid cells during the 1st week of life is the most striking point in Chart 2. By the 4th to the 7th days of life the erythroid cells have reached an average of 70 per cent of the marrow cells. After the 7th day there was a gradual approach of these two strains of cells to approximate equality which was reached in the 4th week of life and maintained for the next 4 months (11, 12).

The counts of the cells of the marrow from the different bones of any animal have been, for the most part, consistent, and the irregularities in the curve of the early stages up to the 7th day have been due to single animals that have varied from the mean. The figures plotted for the 29th and 30th days, each from records of litter mates, showed the erythroid cells to be consistently high, varying from 57 to 82 per cent for the 29th day and from 52 to 86 per cent for the 30th. For one fetal rabbit, R 1170 A, we lacked a record of the date of mating but the litter was near maturity. The differential count is recorded in Table II but not plotted in Charts 2 and 3. The differential count showed a myeloid predominance.

On the day of birth, the counts of the erythroid cells in all 4 animals were close to the average, being 31, 40, 43, and 55 per cent respectively. The fall in the erythroid cells on the 2nd day was due to 2 animals, R 1779 and R 1780, in which the erythroid cells were 18 and 22 per cent respectively, while the other 4 animals had a range of from 42 to 69 per cent. On the 3rd and 4th days, all the counts were near the average; the drop on the 5th day was due to one animal only, R 4377, in which the percentage of erythroid cells was 21, while in the others the range was from 72 to 82 per cent. It is interesting to note that in an animal of the same litter as R 4377, counted the day before, that is on the 4th day of life, the average of erythroid cells was 75 per cent. Thus the drive toward erythroid cells was not initiated in every animal of a given litter at exactly the same age. The counts of the animals on the 6th and 7th days were consistent and near the mean. In general, of the 30 animals whose bone marrow was studied from late fetal stages through the 7th day of life, only 4 showed the erythroid cells below the myeloid.

The period of the 1st week of life is also significant in the preponderance of immature over mature cells in each of the two strains (13). This is shown on Chart 3. The erythroid cells are pictured in the lower half of the chart and it will be noted that, with the exception of the 3rd day, the immature cells were higher than the mature ones up to the 6th day of life. In the records for the 3rd day, the reverse was true for both of the animals counted, which were from the same litter. In the myeloid strain, on the other hand, the mature cells predominated earlier, namely, from the 3rd day onward.

Data from Study of Sections of Bone Marrow

The study of the sections of the bone marrow in these stages adds important facts to those which can be obtained from the study of individual cells. In general, sections of bone marrow provide material from which to estimate the total amount of hematopoietic tissue. In late fetal life, the long bones are small and contain little marrow. The entire shaft contains spicules of bone with osteoclasts upon them.

On the day of birth, the bone marrow is relatively inactive (Fig. 1, from rabbit R 1627). The differential count in this animal was: erythroid cells, 65.8 per cent; myeloid, 18.5 per cent; primitive cells, 14.6 per cent, and accessory types, 1.1 per cent. Spicules of bone were present and the right hand one shows an osteoclast. By the 2nd day (Fig. 2) the spicules of bone had entirely disappeared, cleared away by osteoclasts. Such an osteoclast, shown in Fig. 3, gives significant evidence of its phagocytic function, being filled with particles which stained red in the eosin-methylene blue. On the 2nd day of life, the marrow (Fig. 2) had the sinusoids widely dilated and filled with blood corpuscles. Between the sinusoids the marrow cells were sparse. In this rabbit the differential count was: erythroid cells, 22.6 per cent; myeloid cells, 44.8 per cent; primitive cells, 31.2 per cent, and clasmotocytes and unclassified cells together, 1.3 per cent. The upper part of Fig. 3 is from the same preparation as the osteoclast shown in the lower part. This marrow also had about the same differential count as the one shown in Fig. 2. These 2 animals illustrate the fact that if the bone marrow is still relatively inactive, there may be a myeloid predominance.

Quite different is the proportion of the marrow cells to be seen 3 days later. The beginning of activity in the marrow due to erythroid hyperplasia is illustrated in Figs. 4 and 5 from a rabbit, R 1784, studied on the 5th day of life. Fig. 4 is a section of the tibia of this animal and Fig. 5 is from a film of this marrow stained in Wright's methylene blue-eosin. More than half of the marrow was hyperplastic, as is shown in the lower part of Fig. 4; the rest was like the marrow of the 2nd day, as is clear in the upper part of Fig. 4. The sinusoids were still filled with blood; there were no fat cells. The hyperplasia, which represents the fact that an enormous amount of cell division has taken place in 3 days' time, was due to the erythroid cells. The differential count was 73.2 per cent erythroid cells; 10.1 per cent myeloid; and 16.7 per cent primitive cells.

The further development of the erythroid hyperplasia and its liquidation are shown in Figs. 6 to 9' from sections of bone marrow from the 2nd, 3rd, and 4th weeks of life. During the 2nd week the marrow becomes almost completely hyperplastic and fills the marrow cavity (Fig. 6). During this week erythroid cells were consistently high, the counts being 64, 67, 74, and 77 per cent respectively. This hyperplastic phase of the marrow is transitory, as shown in Figs. 7 to 9'. The rest of the 1st month is characterized by a rapid development of fat cells, which is correlated with a rapid increase in the size of the marrow cavity. These factors serve to reduce the hyperplasia by a marked spacing of the marrow cells. The beginning of the dispersion of the marrow cells is shown in Fig. 7 from rabbit R 1234, studied in the 3rd week of life. The differential count of the marrow cells showed a slight preponderance of myeloid types. The proportions were: erythroid, 34.3 per cent; myeloid, 55.9 per cent; primitive, 9 per cent; and clasmatoocytes, 0.6 per cent. In the section the occurrence of the fat cells is clear.

Figs. 8, 8', 9, and 9' are all from the same animal, R 2048, from the 4th week of life. They show a reduction of the hyperplasia due to the growth of the bones and the consequent increase in the size of the marrow cavity. This stage of the development of the bone marrow represents a natural simplification of this organ which makes it possible to study to advantage the relationships of the cells of the primitive types to the myeloid and the erythroid strains. The connective tissue cells occur in two forms. Scattered throughout the bone marrow are cells of the size and type of the reticular cell or the fibroblast. Their nuclei are oval and have little chromatin; their cytoplasm is branched and shows little reaction to either basophilic or acidophilic stains. Since during the 4th week the fat cells are appearing in great numbers, it is easy to see that some of them become the fat cells. Others remain as fibroblasts. More important, from the standpoint of hematopoiesis, is the type we have recorded under the name primitive cell. This cell more nearly resembles the small lymphocyte than any other type. Several of them are indicated by arrows in Fig. 8'. One is marked by the left arrow in Fig. 9, in which it will be noted that the nucleus is poor in chromatin, showing only as a few tiny granules along the inner border of the nuclear membrane. Another primitive cell is shown as C in Fig. 9. In the living state this cell shows fewer signs of differentiation than the lymphocytes of lymph nodes, spleen, and blood. The cytoplasm of lymphocytes usually contains a few vacuoles which stain with the neutral red. Moreover, mitochondria, usually in the form of rods, are easily seen without any stain and reacting readily to vital Janus green, are characteristic. In the primitive cells, on the other hand, as seen in this material from bone marrow, the narrow rim of cytoplasm contains almost no visible granules of any sort. The cytoplasm has little basophilia, and mitochondrial material must be finely divided. It is probable that in our differential counts of marrow cells we have underestimated the number of primitive cells on account of a tendency to select zones for enumeration in which the major strains of cells are clear and predominant. However, our records show (Chart 2) that up to the 5th day these cells are consistently above 5 per cent.

In supravital and fixed films of these early marrows we have found a few cells of this primitive type containing a few specific neutrophilic granules. They are clear in the sections of the marrow of rabbit R 2048. The primitive cell, to which the upper arrow of Fig. 8' points, contains a few neutrophilic granules, as is clear at higher magnification in Fig. 8. These small granulocytes are fewer than the typical myeloblasts and myelocytes A, but they are important in connection with granulopoiesis, and will be considered in the discussion. The right hand arrow of Fig. 9 points to a myeloblast, which shows the increase in chromatin of the nucleus in this cell from the stage of the primitive cell.

This material from the 4th week of life is also significant for the study of the place of origin of both myeloid and erythroid strains. The extravascular origin of the granulocytes is well established, so the position of primitive cells, myeloblasts, and myelocytes outside the vessels, as shown in Figs. 8' and 9', but illustrates a well known point.

Concerning the red cells, on the other hand, there are marked differences of opinion. In Fig. 9' is shown what we interpret as an erythrocytic capillary containing normoblasts. It lies obliquely across the upper part of the figure; in the upper left hand corner of this section is a mass of late erythroblasts or normoblasts, marked A, lying between two endothelial nuclei. Just to the right of them are two more endothelial nuclei, labeled B, the upper one of them being markedly swollen. Between these two nuclei is some material out of focus which is a clasmocyte along the border of the vessel. Farther to the right, the endothelial wall of the vessel is still evident, with two leucocytes also out of focus, while beyond is an erythroblast, B', at the beginning of a sinusoid, cut off in the photograph. The lower half of the section is occupied by myelocytes, mainly of Type B. One myeloblast in division is seen near the right hand border and near it is a primitive cell, marked C.

Further evidence on the placing of the red cells in bone marrow is shown in Figs. 10 to 13. The upper figures are from the bone marrow of rabbit R 1460, of the 2nd month of life, and the two lower figures from rabbit R 1306 from the 3rd month. Figs. 10 and 12 at the left, taken at low magnification, show that the marrow is not yet as packed with cells as it becomes in adult life. This is also especially marked in the border of the marrow, as shown in Fig. 11. The border later becomes densely packed with myelocytes, but at this stage they are almost lacking and thus the placing of the red cells is unmasked. To the left is a mass of erythroblasts and normoblasts, and the edge of the capillary in which they are contained is marked A. Other masses of normoblasts are marked B. In Fig. 13, a large sinusoid marked A passes obliquely across the field. The endothelial nuclei are clear as well as the contained erythrocytes. In the upper part of the figure is a large mass of erythroid cells, marked B, normoblasts and erythroblasts at what we interpret as the opening of an erythrocytic capillary, while on the lower right corner is a mass of normoblasts separated from the lumen of the sinusoid.

CHANGES IN BONE MARROW AND BLOOD CELLS

DISCUSSION

The most striking point brought out in this study is the speed with which the young rabbit is able to produce the number of red blood cells characteristic of the blood of the adult animal in contrast to the delayed production of the corresponding number of white blood cells. By the 3rd week of life the rabbit's blood has its full quota of red blood cells, while the ultimate number of white blood cells is not reached until the 6th month.

Recent studies of red cells indicate two mechanisms, one for the elaboration of hemoglobin and the other for multiplication of red cells. In the embryo the mechanism for the elaboration of hemoglobin is more efficient than for the manufacture of the cells, but soon after birth the materials which stimulate the multiplication of red cells become available. The study of the bone marrow indicates that as this organ begins to assume function at the time of birth, the predominance of its activity is in the erythroid series, a sign of the imperative need of the body for hemoglobin. Thus the immediate activity of the marrow delivers to the blood stream rapidly increasing numbers of red cells but for the 1st month no increase whatever in granulocytes.

It has long been established that the first strain of blood cells to appear in both avian (14-16) and mammalian (17) embryos is the erythroid; and of mammalian embryos it is known that the white blood cells remain low in the blood stream throughout fetal life. This disproportion is shown in our data of the last few fetal days of the rabbit, where the white blood cells average only 900 per c. mm., or about 10 per cent of the number in the adult, while the 3,000,000 red cells of the same period are more than half their final number.

At the time of birth there is an increase in both strains of cells in the blood stream, probably due to a flooding of cells into the blood vessels from the sinusoids of the liver as its circulation is changed from the fetal type. In the case of the red cells, the increase to the circulation is of a million cells per cubic millimeter. The animals are, however, not able to maintain this increase in red cells during the 1st week of life, due perhaps in part to the sudden cessation of erythroid function in the liver and a lag period before the bone marrow can assume function. Another factor in the fall in red cells during the 1st

week of life may be an increase in blood volume, for which a stable number of red cells, or even a slight increase, cannot compensate.

The bone marrow, on the other hand, shows signs of a remarkable concentration of the factors which stimulate both the multiplication of red cells and the elaboration of hemoglobin. In late fetal stages the marrow is relatively quiescent and usually predominantly erythroid. Immature red cells are in greater numbers than mature ones, so the marrow cannot give many erythrocytes to the circulation. The time of the initiation of erythroid activity varies somewhat even in the same litter; it may start on the day of birth or be delayed until the 4th day, but is in full swing during the last half of the 1st week. This leads to a marked erythroid hyperplasia of the bone marrow during the 2nd week. The activity of the bone marrow from the 5th to the 7th days of life is not adequate to deliver enough mature red cells to the circulation to raise their number on account of the time needed for maturation; but during the 2nd week the marrow succeeds in increasing the red cells in the blood by half a million per cubic millimeter.

The appearance of hyperplasia in the bone marrow during the 2nd week of life means that for a short time the animal has to use much of the available space in the marrow cavity for the production of red cells. In the adult animal there are three mechanisms available to increase space for the formation of blood cells. The first is the shifting of fat from the bone marrow. The fat within the fat cells of the marrow is in a labile state; reversing the method of its formation, the fat readily breaks up into small droplets, leaves the cells, and passes into the blood stream. The second method is a thinning of the bone to make the cavity larger. During the 2nd week of life it is the growth of the bone and consequent enlarging of the marrow cavity that is the major factor in the adjustment. This means that the control of the growth of bone in young animals has a bearing on erythropoiesis. The cause of this adjustment must be chemical and perhaps of the nature of an endocrine balance; but one factor in the method for increasing the size of the marrow cavity is the formation of osteoclasts along its edge. It may be that a marked crowding of the marrow may favor the fusion of the osteoblasts into the giant cells that erode the bone. The mechanism for bone regression as well as for bone growth

is, of course, cellular. The rapid development of fat cells in the bone marrow during the 3rd and 4th weeks of life in the rabbit occurs during a period of rapid growth of the bone. The third method of increasing space for the formation of blood cells is the use of extramedullary zones.

From these observations it is clear that the first 3 weeks of life are crucial for the study of red cells in the rabbit. During this period the specific substances for the multiplication of red cells and the building up of hemoglobin must be present in high concentration. This mechanism is correlated with the factors that control the growth of bone. Thus the erythroid hyperplasia, which was built up so rapidly, is reduced almost as quickly, as can be seen by comparing Fig. 6 from the 2nd week with Fig. 8' from the 4th.

One of the differences in opinion concerning the development of blood cells at the present time, has to do with the place of origin of the red cells. It is in general agreed that in early embryonic stages in both birds and mammals the red cells arise within the vessels (1, 14-16). In mammalian forms, Maximow (17) judged that in the adult stages the evidence indicates that the red cells develop extravascularly and, when mature, break through the walls of the vessels to enter the blood stream. Recently, Jordan and Johnson (18) have expressed the same view for the adult pigeon. On the other hand, Doan (19, 20) and Doan, Cunningham, and Sabin (21) consider that the evidence indicates that in both avian and mammalian bone marrow red cells arise throughout life in collapsed capillaries. The opening of these erythrogenic capillaries into the sinusoids when erythrocytes are mature is the most feasible theory yet formulated for the entrance of these cells into the circulation. This is true since the erythrocyte does not possess the power of active locomotion. In the simplified marrow of the 4th week it is easy to find masses of developing red cells which appear to be surrounded by walls that may be interpreted as endothelial borders. The point cannot be made out for every group of developing red cells, especially when red cells are closely placed against myelocytes. This seems to us to be inevitable since in no other organ can the full capillary bed be determined without injection.

This study of the development of the white blood cells has brought out the fact that the leucopenia characteristic of fetal stages is overcome slowly by the rabbit and disappears only by the 6th month of

Our data on lymphocytes and monocytes show that their numbers increase rapidly in the blood stream during the 1st of month of life. Thus neither strain shows any depression from the predominance of erythropoiesis, such as is suffered by the white blood cells which develop in the marrow. The monocytes, coming as they do from the primitive cells diffusely scattered in the connective tissues, are not affected by the changes that take place in the marrow at birth and are the first cells of the blood stream to reach their full quota. This is accomplished in the 1st week of life so that they even precede the red blood cells in reaching maturity. Our studies of the blood cells indicate that the lymph nodes of the rabbit develop rapidly toward functional activity during the 1st month of life and then more slowly for the next 5 months.

SUMMARY

1. The full number of erythroid cells in the blood stream of the rabbit is reached by the 3rd week of life.

2. During this period, there is a predominance of erythrogenesis in the bone marrow.

3. During the 2nd week of life the bone marrow is in a state of hyperplasia owing to the needs of the body for blood and the small space available for the marrow.

4. This hyperplasia is reduced as the growth of the bone permits the marrow to spread. The control of the growth of the bones has an important bearing on hematopoiesis.

5. During the first 3 weeks of life, the chemical factors for the multiplication of red cells as well as for the elaboration of hemoglobin become available.

6. The amount of hemoglobin does not increase as rapidly as the number of cells, so that the macrocytic anemia of the fetus becomes reduced. The proportion of hemoglobin per red cell characteristic of the adult rabbit is reached by the 3rd month.

7. Further evidence on the intravascular origin of red blood cells is given.

8. The development of all of the white blood cells, with the exception of the monocyte, goes on at a slower rate than that of the red cells.

9. The monocytes reach their full number in the blood stream in

tive myelocytes. In 1929, Smith and McDowell (23) described the occurrence of such small basophilic leucocytes in normal human blood and we have seen tiny granulocytes with neutrophilic granules in rabbits' blood. On the other hand, the primitive cell which has elaborated a few granulations may then enlarge and become a typical myelocyte.

It is quite clear that the occurrence of any granulations in these primitive types may be interpreted as evidence of the origin of granulocytes from lymphocytes (24-29) and we do not wish to stress a difference without a distinction, nor one which is merely to be resolved by terminology. The real distinction concerns the matter of potentialities of lymphocytes. In the one theory the so called primitive cell is undifferentiated and has all the potentialities for developing into any form of blood cell, while the lymphocyte, as seen in lymph nodes, spleen, and in the blood stream, is a mature cell with a distinct functional rôle. If the lymphocyte is a mature functioning cell, with as definite a cycle of maturation as the granulocytic strains (30, 31), it is in the interests of clarity to keep the term primitive cell for the less differentiated type. If, on the other hand, the mature lymphocyte can function for a time as a lymphocyte and then become either a granulocyte or a monocyte, then the lymphocyte must be considered both as a mature functioning cell and as a stem cell at the same time. The latter seems to us improbable.

As is shown on Chart 2, the primitive cell occurs in greater proportion around the time of birth than in the normal marrow of later stages. In still earlier fetal stages, the primitive cell is the predominating type, giving a marrow which Maximow (1) named primary lymphoid marrow. Correlated with these facts, certain observations on pathological bone marrows from work in progress by Dr. C. P. Rhoads and Dr. D. K. Miller at The Rockefeller Institute are of great interest. We are permitted by them to state them as follows: Under three conditions the primitive cell becomes a prominent feature of the bone marrow, not because it has increased in numbers but because the more differentiated cells have disappeared; second, in agranulocytosis, and third, in certain cases of anemia with leucopenia, primitive cells become increased in numbers.

Our data on lymphocytes and monocytes show that their numbers increase rapidly in the blood stream during the 1st of month of life. Thus neither strain shows any depression from the predominance of erythropoiesis, such as is suffered by the white blood cells which develop in the marrow. The monocytes, coming as they do from the primitive cells diffusely scattered in the connective tissues, are not affected by the changes that take place in the marrow at birth and are the first cells of the blood stream to reach their full quota. This is accomplished in the 1st week of life so that they even precede the red blood cells in reaching maturity. Our studies of the blood cells indicate that the lymph nodes of the rabbit develop rapidly toward functional activity during the 1st month of life and then more slowly for the next 5 months.

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Our data on lymphocytes and monocytes show that their numbers increase rapidly in the blood stream during the 1st of month of life. Thus neither strain shows any depression from the predominance of erythropoiesis, such as is suffered by the white blood cells which develop in the marrow. The monocytes, coming as they do from the primitive cells diffusely scattered in the connective tissues, are not affected by the changes that take place in the marrow at birth and are the first cells of the blood stream to reach their full quota. This is accomplished in the 1st week of life so that they even precede the red blood cells in reaching maturity. Our studies of the blood cells indicate that the lymph nodes of the rabbit develop rapidly toward functional activity during the 1st month of life and then more slowly for the next 5 months.

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8. The development of all of the white blood cells, with the exception of the monocyte, goes on at a slower rate than that of the red cells.
9. The monocytes reach their full number in the blood stream in

the 1st week of life; granulocytes and lymphocytes by the 5th and 6th months.

10. Each of the three strains of white cells has a different rate of development.

11. The question as to whether the stem cell or primitive cell is identical with the lymphocyte is discussed.

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EXPLANATION OF PLATES

PLATE 7

FIG. 1. Section of the bone marrow of the tibia of rabbit R 1627, day of birth, stained with Giemsa. It shows two spicules of bone and marrow cells between. The differential count of the marrow of this animal was erythroid cells, 65.8 per cent; myeloid cells, 18.5 per cent; primitive cells, 14.6 per cent; accessory types, 1.1 per cent. The left arrow passes between some mature erythrocytes and points to a group of nucleated red cells. The right arrow points to an osteoclast. $\times 180$.

FIG. 2. Section of the bone marrow of the humerus of rabbit R 1780, 2nd day of life, stained with hematoxylin and eosin. The differential count of the marrow of this animal was erythroid cells, 22.6 per cent; myeloid cells, 44.8 per cent; primitive cells, 31.2 per cent; and clasmatoocytes and unclassified together, 1.3 per cent. $\times 240$.

FIG. 3. Two parts of a fixed film of bone marrow of rabbit R 1779, litter mate of preceding animal, 2nd day of life, stained with Wright's eosin-methylene blue. The lower half of the figure shows an osteoclast filled with granules which stained red and were probably phagocytized bone. On the lower part of the osteoclast is a small cell out of focus. The upper part of the figure shows a marrow predominantly myeloid. Six normoblasts are plain and the rest are myeloid cells. The differential count was erythroid cells, 19.3 per cent; myeloid cells, 67.7 per cent; primitive cells, 12.7 per cent; and accessory types, 0.2 per cent. $\times 750$.

FIG. 4. Section of the bone marrow of the tibia of rabbit R 1784, 5th day of life, stained with hematoxylin and eosin. The differential count was erythroid cells, 73.2 per cent; myeloid cells, 10.1 per cent; primitive cells, 16.7 per cent. $\times 240$.

FIG. 5. Fixed film of bone marrow of the same rabbit as in Fig. 4, stained with Wright's eosin-methylene blue. All but three of the cells are erythroid. A is a clasmatoocyte containing three normoblasts; B is a myelocyte B; C is a myeloblast. $\times 1,000$.

PLATE 8

FIG. 6. Section of the bone marrow of the femur of rabbit R 1466, 13th day or 2nd week, stained with hematoxylin and eosin. It shows a hyperplastic state. The differential count of this animal was erythroid, 79.5 per cent; myeloid, 19.5 per cent; primitive cells, 0.5 per cent; and accessory cells, 0.5 per cent. $\times 240$.

FIG. 7. Section of the bone marrow of rabbit R 1234, 3rd week of life, stained with hematoxylin and eosin. The differential count of the marrow of this animal was erythroid cells, 34.3 per cent; myeloid cells, 55.9 per cent; primitive cells, 9

per cent; clasmotocytes, 0.6 per cent. The fat cells are plain in the clear spaces. $\times 240$.

FIG. 8. Part of the same section as in Fig. 8', at a magnification of 1,200 diameters, to show a primitive cell containing a few neutrophilic granules in the cytoplasm. It is marked with an arrow and is the same cell as the one marked with the upper arrow of Fig. 8'. The cell marked *a* is similarly marked in Fig. 8' and may be an endothelial cell against a mass of normoblasts.

FIG. 8'. Section of the bone marrow of the tibia of rabbit R 2048, 4th week of life, stained in Giemsa. Section is 4μ thick. The differential count in this animal was erythroid cells, 23.4 per cent; myeloid cells, 74.9 per cent; primitive cells, 0.3 per cent; and accessory cells, 1 per cent. *A* marks a group of normoblasts; the arrows point to primitive cells. $\times 350$.

FIG. 9. Part of the section shown in Figs. 8 and 8' but from a different area. It shows a typical primitive cell, marked by an arrow. The nucleus has tiny granules of chromatin against the membrane. The right arrow points to a myeloblast. $\times 1,200$.

FIG. 9. Section of the bone marrow of the humerus of the same rabbit as in Fig. 8, stained with hematoxylin and eosin. Section about 8μ thick. *A* is opposite a group of normoblasts between two endothelial nuclei; *B* opposite two endothelial nuclei of the same erythrocytic capillary, the upper nucleus is swollen. The dark cells within the capillary just to the right are leucocytes out of focus. *B'* is an erythroblast. *C* is opposite a primitive cell and to the right of it is a myeloblast in division. In the lower half of the figure are myelocytes. $\times 1,000$.

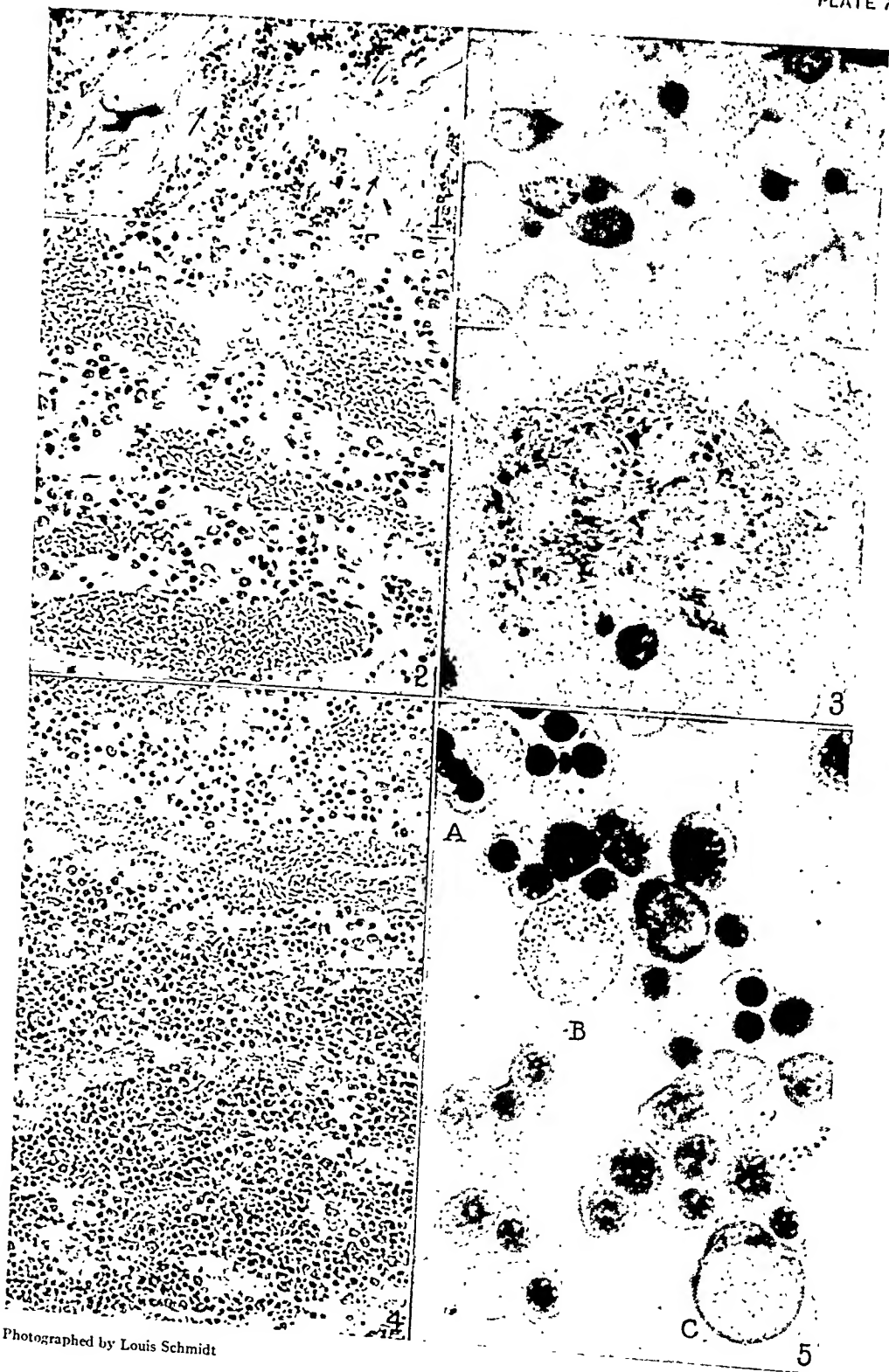
PLATE 9

FIG. 10. Section of the bone marrow of the femur of rabbit R 1460, 2nd month of life, stained with hematoxylin and eosin. The differential count in this animal was erythroid cells, 73.4 per cent; myeloid cells, 24.2 per cent; primitive cells, 2 per cent. $\times 240$.

FIG. 11. Section of the bone marrow of the femur of the same animal as in Fig. 10. *A*, endothelial wall around a group of normoblasts and erythroblasts; *B*, groups of normoblasts. $\times 1,000$.

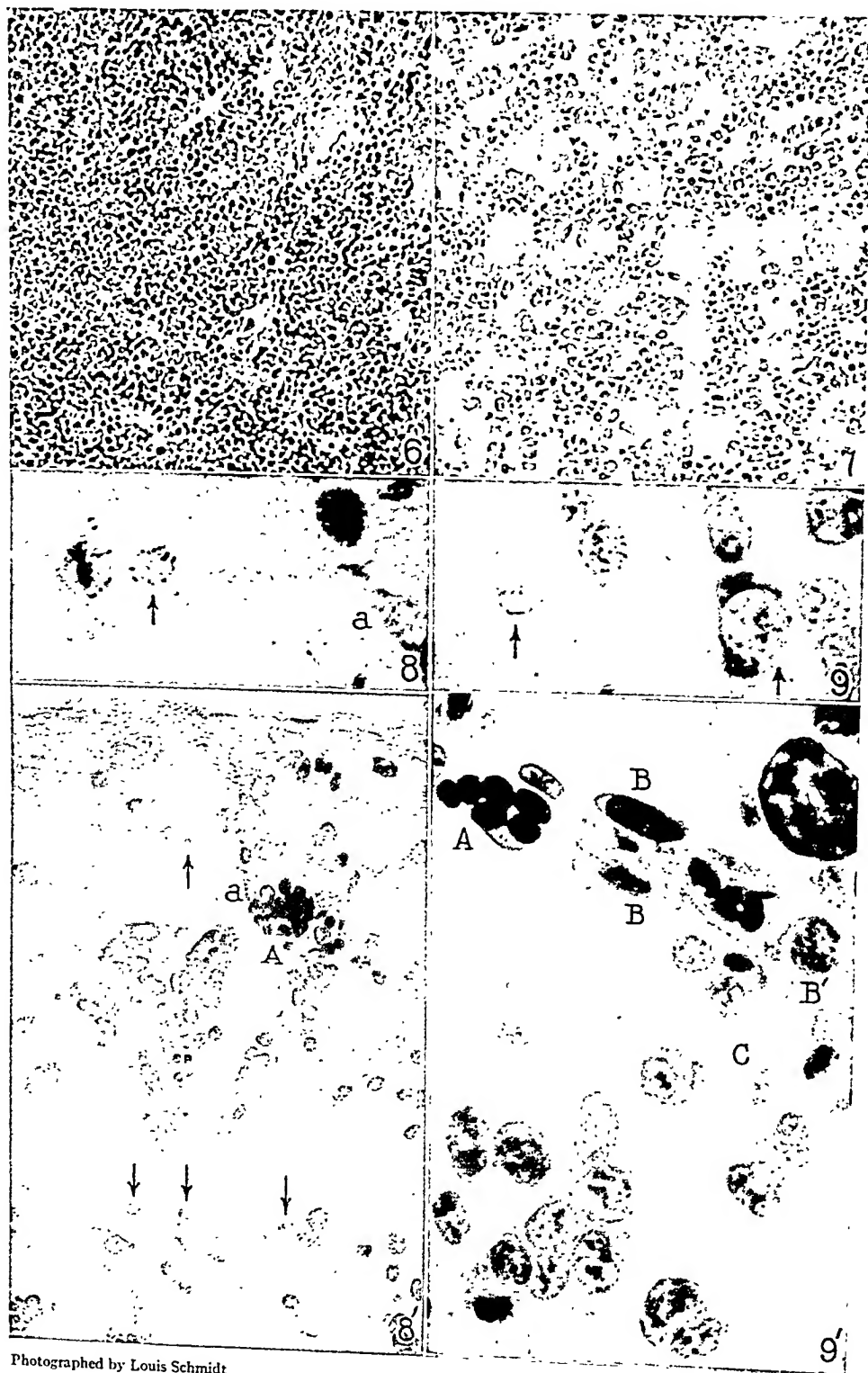
FIG. 12. Section of the bone marrow of the femur of rabbit R 1306, 3rd month of life, stained with hematoxylin and eosin. The differential count of this marrow was erythroid cells, 34.4 per cent; myeloid cells, 64.7 per cent; primitive cells, 0.6 per cent; and megalokaryocytes, 0.3 per cent. $\times 240$.

FIG. 13. Section of the bone marrow of the femur of the same animal as in Fig. 12. Stained with hematoxylin and eosin. The letter *A* is in the lumen of a sinusoid; the upper letter *B* is opposite a mass of nucleated red cells in what we interpret as the opening of an erythrocytic capillary into the sinusoid. The lower letter *B* is a mass of nucleated red cells separated from the sinusoid by an endothelial wall. $\times 1,000$.



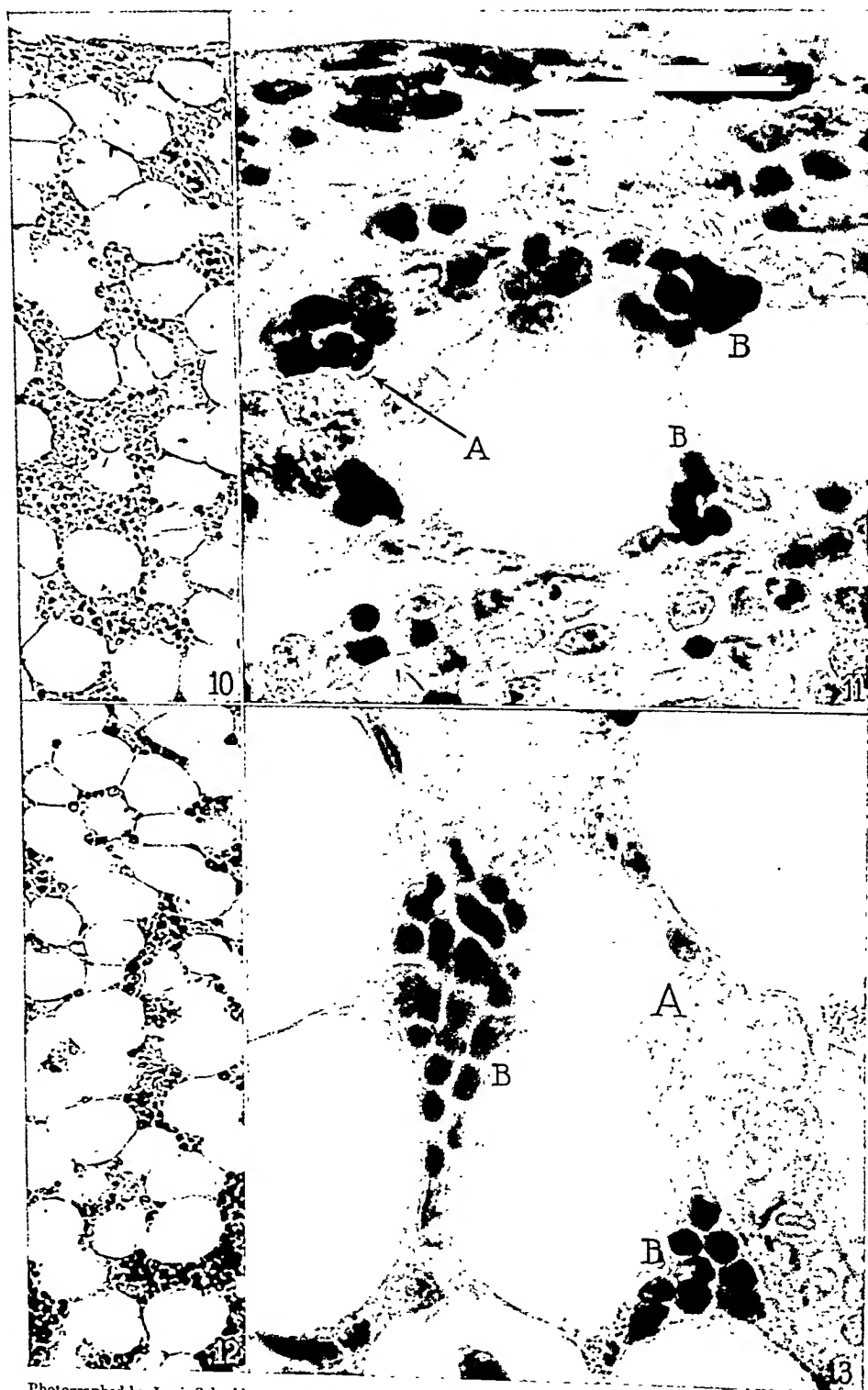
Photographed by Louis Schmidt

(Sabin *et al.*: Changes in bone marrow and blood cells)



Photographed by Louis Schmidt

(Sabin *et al.*: Changes in bone marrow and blood cells)



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(Sabin *et al.*: Changes in bone marrow and blood cells)

THE CULTIVATION OF TISSUES FOR PROLONGED PERIODS IN SINGLE FLASKS

By RAYMOND C. PARKER, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 10 TO 12

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Three years ago, it was reported¹ that common connective tissue cells, or fibroblasts, could be kept alive in Carrel flasks without transferring them for very long periods. At that time, a single culture in its tenth passage had been retained in the same flask for 128 days. The nutrient materials were supplied periodically in the form of serum. Shortly thereafter, however, the culture, while apparently still alive, was lost by infection. It seemed desirable to repeat the experiment in order to determine whether such a culture could be retained in a living state under these conditions for a longer period, and also to study the effect of such treatment on the behavior and fate of the constituent cells. It is the purpose of this communication to report two series of such experiments. In the second of these, various tissue fragments have been kept alive in single flasks for an entire year.

The Cultivation of Tissue Fragments from Skeletal Muscle for 194 Days in Single Flasks

In these experiments, fragments of skeletal muscle from a 16 day old chick embryo were cultivated in three separate flasks. The tissues were embedded in a coagulum comprised of adult fowl plasma and Tyrode solution. After a few days, the first culture received a mixture consisting of 30 per cent adult fowl serum in Tyrode solution, and the second, 50 per cent adult fowl serum in Tyrode solution. Three times a week for the first 50 days, and twice a week thereafter, these mixtures were aspirated and replaced with fresh material. On each of these occasions, the third culture was treated for 2 hours at 37°C. with a mixture of 50 per cent adult fowl plasma in Tyrode solution. The plasma was maintained in a fluid state over this period by the addition of heparin. After 2 hours, the heparinized plasma mixture was removed and the culture left without fluid until the next treatment a

¹ Parker, R. C., *J. Exp. Med.*, 1933, 58, 97.

few days later. The types of handling outlined were continued in all three cases for 194 days, during which time the tissues were not removed from the flasks in which they had originally been placed.

In the beginning, the cultures showed considerable cell multiplication in the marginal areas. As time went on, this became less and less marked. By the 15th day, the cultures had already shown their greatest relative increase in surface area. Also, the peripheral cells became more and more distended and granular until finally they seemed quite lifeless. Eventually, however, new cells appeared among the dead remnants, but after a time these too degenerated, only to be overrun later by an entirely fresh crop that came out from the interior as had those before them. 3 months after the cultures were made, the one treated with heparinized plasma showed a vigorous production of macrophages² that soon covered the entire bottom surface of the flask.

At the end of the 194 day period, the cultures were removed and cut into fragments that were transferred to fresh flasks, with new media consisting of plasma and Tyrode solution. The plasma-Tyrode mixture was allowed to coagulate spontaneously, without the addition of any other substance. After a lag period of from 24 to 72 hours, young, active cells of the so called fibroblast type began to migrate from each of the implanted fragments until finally a dense fringe of new growth had been established (Fig. 1). This served to demonstrate that the inner portions of the original cultures had remained alive. When the cultures had been carried for 6 days in this passage, they were again removed, this time for fixation, sectioning, and staining. The stained sections showed the central areas of the various fragments to consist of a compact, fibrous matrix with an even distribution of well preserved nuclei (Figs. 2-6). No distinct differences were found between the cultures that had been treated with 30 per cent serum and those treated with 50 per cent serum and 50 per cent heparinized plasma. In sections stained according to Masson's trichrome procedure,³ the central areas appeared brilliant green, whereas the peripheral fringe of young cells that had grown out from these areas was invariably red.

² Parker, R. C., *J. Exp. Med.*, 1932, 55, 713.

³ Foot, N. C., *Stain Technology*, 1933, 8, 101.

The Cultivation of Tissue Fragments from Breast Muscle for One Year in Single Flasks

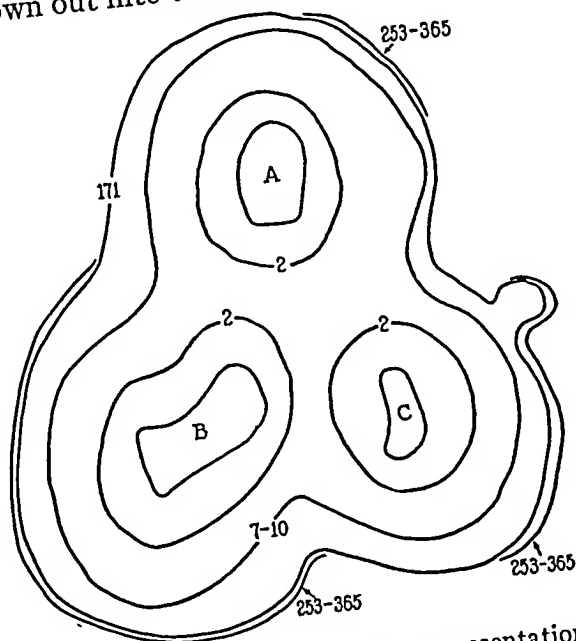
In these experiments, fragments of breast muscle from a 12 day old chick embryo were cultivated in single flasks for a year. One group of fragments was carried in a fluid medium comprised of serum and Tyrode solution. In this case, the only plasma employed consisted of a minute capillary drop that was used to fix the tissue fragments to the bottom of the flask on the day the culture was made. Thereafter, the treatment consisted solely of regular applications of fresh serum diluted with two parts of Tyrode solution. This fluid was changed three times a week for the first 3 months, and twice a week for the remainder of the year.

The other cultures belonging to the series were prepared by embedding the tissue fragments in a coagulum consisting of plasma and Tyrode solution. On three different occasions throughout the year, this coagulum was reinforced by the addition of fresh plasma and Tyrode solution. Otherwise, the nutrient materials were supplied regularly in the form of serum diluted with two parts of Tyrode solution. At the time of treatment (*i.e.*, two or three times a week, as indicated above), this fluid was aspirated, and the cultures were washed for a couple of hours with Tyrode solution and then supplied with a fresh serum mixture. On each occasion, the serum mixture was allowed to remain on the cultures until the next treatment, when the process was repeated.

In all cases, the cultures showed a greater relative increase in surface area during the first few days than at any subsequent period throughout the year. Thus, to take an example, a group of three tissue fragments were embedded in a plasma coagulum and treated with frequent applications of fresh serum (Text-fig. 1). There was a rapid outward migration of cells up to the 7th day, but none whatsoever between the 7th day and the 10th. Also, between the 171st day and the 253rd day, there was a slight increase in surface area but no change thereafter. In other words, on the 365th day, when the culture was removed from the flask, the area of the colony was precisely what it had been $3\frac{1}{2}$ months before.

As in the case of the earlier experiments, there were long periods in which certain peripheral areas consisted solely of dead cells. Eventually, however, these necrotic areas became inhabited again by young, healthy cells that migrated out from within. The appearance of a given marginal area of one of the cultures was recorded photographically at the end of the 1st, 2nd, and 3rd months (Figs. 7-9). At the end of the 1st month (Fig. 7), the structures comprising this area were completely devoid of every manifestation of life. Although

they retained something of their original cell form, they were actually nothing more than lifeless remnants of cells that had long since succumbed. When the next photograph was taken at the end of the 2nd month (Fig. 8), the appearance of the entire area was almost precisely what it had been before. The same partially disintegrated structures were observed on the same points of the margin. At the end of the 3rd month, however, a phenomenal change had taken place (Fig. 9). New cells had grown out into the area from the interior of the culture.



TEXT-FIG. 1. Culture 5989-2. Diagrammatic representation of the increase in area of three fragments of breast muscle isolated from a 12 day old chick embryo, and cultivated in adult fowl serum for 1 year without being transferred. These fragments were embedded in a plasma coagulum. (Cf. Fig. 11.)

But instead of displacing or destroying the fragmented remains of the earlier cell population, these new cells mingled with them and moved out beyond them, thereby forming new marginal areas (Fig. 10). This process repeated itself at irregular intervals over long periods. Peripheral areas that had been dead and inactive for months would suddenly be filled with new life, as it were. Sometimes, these regions of renewed activity would be extremely localized with the result that peninsula-like contours would be formed (Text-fig. 1). At other times, the entire margin would be involved.

When the various cultures had been treated according to these procedures for exactly 1 year they were removed from their respective flasks, cut into small fragments, and the fragments transferred to fresh flasks and new medium. The medium consisted of a semisolid coagulum comprised of adult fowl plasma and Tyrode solution. Again, as in the case of the cultures carried in single flasks for 194 days, active growth resulted (Fig. 11). The lag period was somewhat longer than before and lasted from 2 to 4 days. Eventually, however, the fragments were completely surrounded by new marginal cells of the so called fibroblast type. Additional nutrient materials were supplied by three subsequent applications of adult fowl serum diluted with Tyrode solution. On the 10th day, the subcultures were fixed in Zenker-formol, after which sections were cut and stained according to the trichrome procedures of Masson³ (Figs. 12-16; 18-20).

As already indicated, one of the original cultures had been carried for 1 year in a flask devoid of the usual plasma coagulum. This particular culture, in common with the others belonging to the series, received regular nourishment in the form of fresh serum. By subculturing the material at the end of the year (Fig. 17), it was discovered that the central portions of the tissue mass contained an even distribution of living cells. But the stained sections that were prepared from the subcultures disclosed even more. The innermost regions of the tissue mass were found to contain a considerable quantity of well formed connective tissue fibers (Fig. 18). After treatment with the Masson stains, these fibers appeared brilliant green. Very often, they were arranged in wavy, parallel bundles. In addition, the cultures contained a dense accumulation of sharply defined muscle fibers (Figs. 19 and 20), in many of which it was still possible to detect cross striations and myofibrillae. Unfortunately, however, it has not been possible to reproduce these striations photographically.

In other cultures belonging to the series, *i.e.*, those embedded in a plasma coagulum, no muscle fibers were present; but the connective tissue was particularly well developed. In the central areas of the original explants (Fig. 12), the nuclei were usually larger and more numerous than at the periphery (Figs. 13 and 15). In all cases, the matrix in which they were embedded was distinctly fibrous. The cytoplasm of the cell bodies was never discernible. Towards the mar-

gin of certain areas, the nuclei were completely absent and the fibers were packed together in the form of dense, parallel bundles (Fig. 14). There were also certain intermediate zones in which the connective tissue fibers were shorter and more irregular in their arrangement (Figs. 13, 15, and 16). In these areas, the nuclei were usually small, abnormal in appearance, and few in number.

DISCUSSION

In the present experiments, fragments of breast muscle from an embryonic chick have been kept alive for a year without disturbing them in any way except to change, periodically, the nutrient fluids that bathed them. It is believed that this is the longest time that animal tissue cells, cultivated *in vitro*, have been maintained in a living state without being transferred. The nutrient fluids consisted of adult fowl serum diluted with Tyrode solution. The techniques that proved effective are exceedingly simple. They require the minimal quantity of materials and but one item in full measure, namely, a strict observance of asepsis. In fact, it is just this simplicity that has been largely responsible for the present report, since the basic procedures can be so modified as to be extremely useful in many different connections, and with little or none of the organization and complications required for the isolation and care of tissue cells in pure strains.

As is usually the case where fixed tissues are involved, the effect of the serum was almost invariably injurious to the marginal cells. Although the fluid medium was changed at frequent intervals, the great majority of the peripheral cells gradually degenerated and finally died. From time to time, however, and even in instances in which the cells of a given marginal area had been dead for several months, new cells would suddenly begin to migrate out from the interior. Occasionally these cells would multiply to form a broad band of new growth out beyond the dead margin, and at other times, the area of this new activity would be quite localized. It was difficult, of course, to account for these sudden spurts of activity. It is probable that they may have corresponded to periods during which a particular sample of serum was being applied. Such a serum may have contained fewer inhibiting substances than usual. But whatever may have been the

cause of these irregularities, the fact remains that the gross appearance of the tissue mass bore little or no relationship to the conditions existing within. For, although the majority of the cultures appeared dead and degenerate for months, the central regions of the tissue masses continued to live for an entire year. At the end of this period, the area of outgrowth from the various fragments was only slightly greater than that attained at the end of the 7th day. It would seem, then, that an appropriate balance had been established between cell multiplication and cell death in which both had been reduced to a minimum.

The present experiments have also demonstrated that fragments of breast muscle cultivated in serum remain alive regardless of whether they are embedded in a plasma coagulum or simply placed in the bottom of the flask and left exposed to the direct action of the serum. Tissues cultivated under both conditions were equally viable at the end of the year. There was also, in each case, an abundant production of adult connective tissue. At the end of the year, however, the fragments that were cultivated in a fluid medium were further characterized by a dense accumulation of muscle fibers. These were completely absent from the tissues that had been embedded in plasma. But the plasma cultures showed an even greater abundance of connective tissue fibers. At present, there is no way in which to account for these differences. The most that can be said is that the fluid medium seemed to favor the retention of muscle elements, whereas the plasma medium favored the production of connective tissue. Levi⁴ is of the opinion that de-differentiated myoblasts may produce collagenous connective tissue fibers. It is possible that this has taken place in the present experiments. If such was the case, then the plasma cultures may have produced more connective tissue than the fluid cultures because in the former the de-differentiation of muscle was complete. Be that as it may, the experiments have served to demonstrate one point conclusively, namely, that the presence of a plasma coagulum is absolutely unnecessary to the continued survival and further development of tissues cultivated in serum.

The fact that connective tissue fibers may develop *in vitro* has been

⁴ Levi, G., *Z. ges. Anat.*, 1934, 31, 471.

shown by many investigators. But the exact manner in which the fibers arise is still obscure. Baitzell⁵ and von Möllendorff,⁶ both of whom worked with plasma cultures, have set forth the theory that they arise by a simple modification of the fibrin contained in the plasma coagulum of the medium. Lewis,⁷ Maximow,⁸ Bloom,⁹ McKinney,¹⁰ Huzella,¹¹ Grossfeld,¹² Momigliano-Levi,¹³ Olivo,¹⁴ and Doljanski and Roulet,¹⁵ on the other hand, hold this to be unnecessary. In fact, three of these workers (Lewis, Grossfeld, and Olivo) have demonstrated that collagenous fibers may be produced by cells cultivated in a fluid medium in the complete absence of preformed fibrin. This has been confirmed in the present experiments. It is apparent, therefore, that the formation of collagenous fibers is a direct manifestation of cellular activity, and may take place in almost any type of medium that will keep the cells alive, and that coagulated plasma is certainly not required for the development of connective tissue fibers *in vitro*.

SUMMARY AND CONCLUSIONS

1. Fragments of breast muscle from a 12 day old chick embryo have been kept alive in single flasks for an entire year without being transferred. The nutrient materials were supplied by frequent applications of adult fowl serum diluted with Tyrode solution.
2. When fragments of fixed tissues are cultivated in serum, cell multiplication and cell death are both reduced to an extremely low level.

⁵ Baitzell, G. A., *J. Exp. Med.*, 1915, **21**, 455; *Am. J. Physiol.*, 1917, **44**, 109.

⁶ von Möllendorff, W., *Z. Zellforsch. u. mikr. Anat.*, 1932, **15**, 131.

⁷ Lewis, M. R., *Carnegie Institution of Washington, Pub. No. 226*, 1917, **6**, 45.

⁸ Maximow, A., *Z. mikr.-anat. Forsch.*, 1929, **17**, 625.

⁹ Bloom, W., *Arch. exp. Zellforsch.*, 1929-30, **9**, 6.

¹⁰ McKinney, R. L., *Arch. exp. Zellforsch.*, 1929-30, **9**, 14.

¹¹ Huzella, T., *Arch. Entwcklungsmechn. Organ.*, 1929, **116**, 430; *Arch. exp. Zellforsch.*, 1931, **11**, 170.

¹² Grossfeld, H., *Z. Zellforsch. u. mikr. Anat.*, 1932, **16**, 432.

¹³ Momigliano-Levi, G., *Z. Zellforsch. u. mikr. Anat.*, 1932, **16**, 389.

¹⁴ Olivo, O. M., *Boll. Soc. ital. Biol. sper.*, 1933, **8**, 589.

¹⁵ Doljanski, L., and Roulet, F., *Virchows Arch. path. Anat.*, 1933, **291**, 260.

3. The presence of a plasma coagulum is not essential to the continued survival and further development of tissues cultivated in serum.

4. The fibrinogen, prothrombin, and fibrin of coagulated plasma are not essential to the development of connective tissue fibers *in vitro*.

EXPLANATION OF PLATES

PLATE 10

FIG. 1. Culture 5984-2. A growth of new cells spanning two fragments of tissue excised from a culture of chick embryo skeletal muscle that had been embedded in a plasma coagulum and treated with 50 per cent adult fowl serum for 194 days in a single flask before being transferred to a new medium of like constitution. $\times 115$.

FIG. 2. Culture 5984-2. A section through one of the original tissue fragments shown in Fig. 1. Fixed in Carnoy solution after 6 days in the new medium, and stained according to Masson's trichrome procedure. The sharply defined marginal fringe represents the new cells that have migrated out from the periphery of the original fragment over the 6 day period. $\times 230$.

FIG. 3. Culture 5984-2. Another marginal area from the culture shown in Fig. 2. $\times 620$.

FIG. 4. Culture 5984-5. A section through the center of a tissue fragment cultivated in 30 per cent serum for 194 days in a single flask. $\times 620$.

FIG. 5. Culture 5984-5. Another area from the culture shown in Fig. 4. $\times 620$.

FIG. 6. Culture 5986-2. A section through the marginal area of a tissue fragment cultivated in 50 per cent heparinized plasma for 194 days in a single flask. $\times 230$.

PLATE 11

FIG. 7. Culture 5989-3. A group of degenerating cells at the margin of a culture of chick embryo breast muscle that had been embedded in a plasma coagulum and treated with adult fowl serum for 1 month without being transferred. $\times 115$.

FIG. 8. Culture 5989-3. The area shown in Fig. 7 at the end of the 2nd month. $\times 115$.

FIG. 9. Culture 5989-3. The area shown in Figs. 7 and 8 at the end of the 3rd month. $\times 115$.

FIG. 10. Culture 5989-3. The area shown in Figs. 7, 8, and 9 at the end of the 5th month. $\times 115$.

FIG. 11. Culture 6494-2 (from Culture 5989-2). A growth of new cells from a fragment of tissue excised from a culture of chick embryo breast muscle that had been embedded in a plasma coagulum and treated with adult fowl serum for 1 year before being transferred to a new medium. $\times 21$.

FIG. 12. Culture 6494-4. A section through the center of a tissue fragment that had been embedded in a plasma coagulum and treated with adult fowl serum

for 1 year in a single flask. Fixed in Zenker-formol and stained according to Masson's trichrome procedure. $\times 620$.

PLATE 12

FIG. 13. Culture 6494-4. Another area from the culture shown in Fig. 12. $\times 620$.

FIG. 14. Culture 6494-4. Another area from the culture shown in Figs. 12 and 13. $\times 620$.

FIG. 15. Culture 6494-5. A marginal area from a culture belonging to the series shown in Figs. 12-14. $\times 620$.

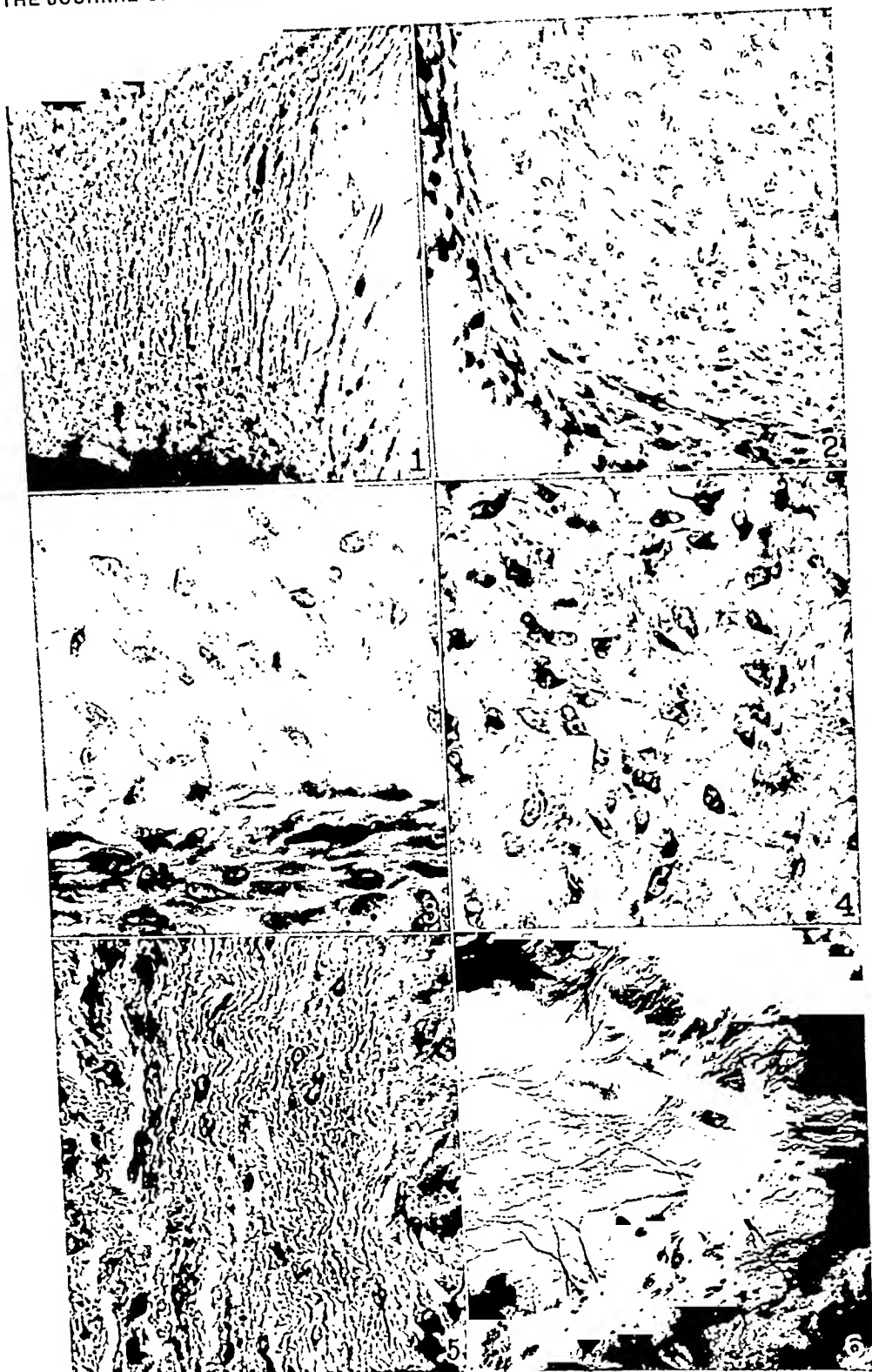
FIG. 16. Culture 6494-4. A central area from the culture shown in Figs. 12-14. $\times 620$.

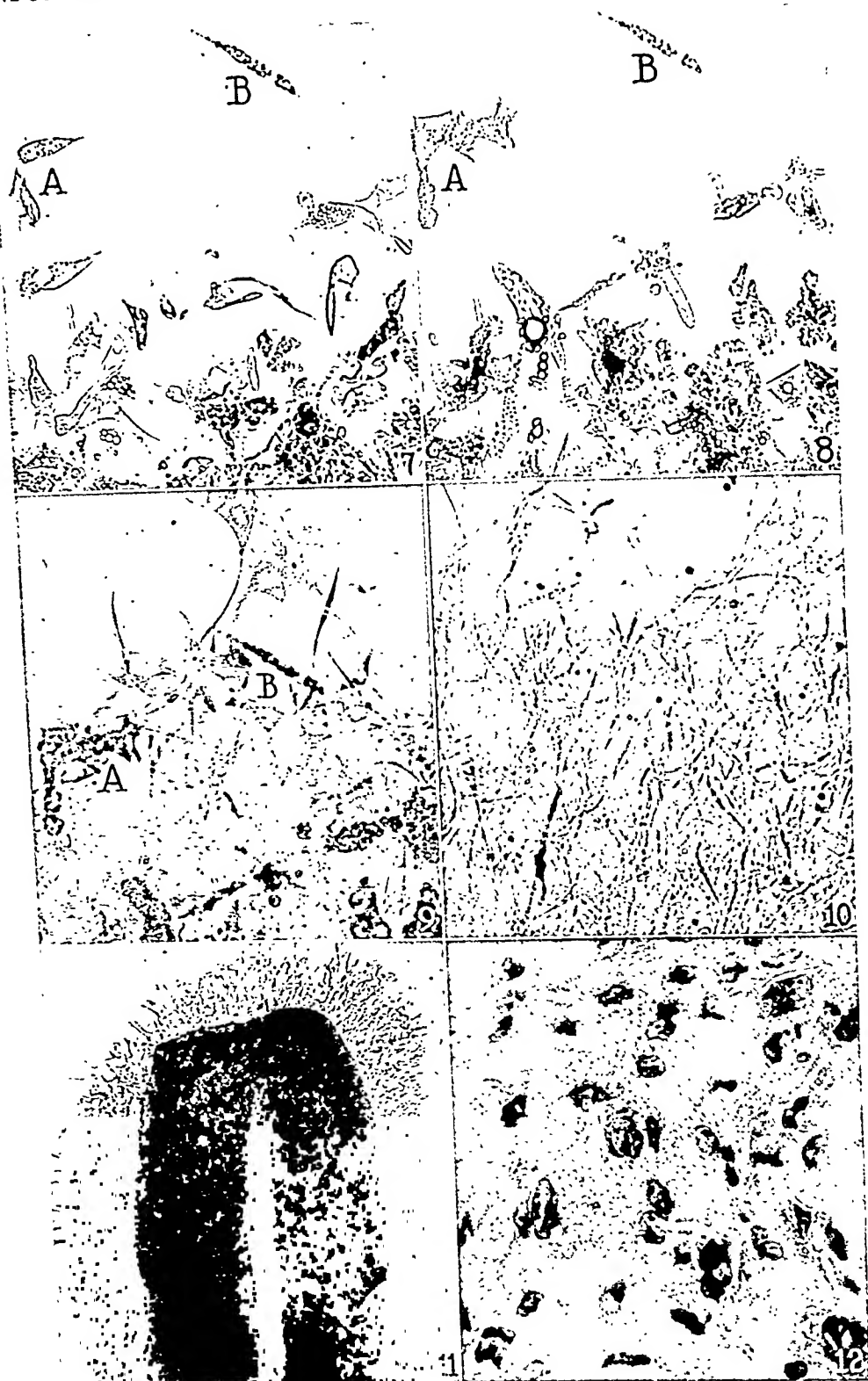
FIG. 17. Culture 6490-1 (from Culture 5990). A growth of new cells spanning two fragments of tissue excised from a culture of chick embryo breast muscle that had been grown in a fluid medium consisting of adult fowl serum for 1 year before being transferred. $\times 115$.

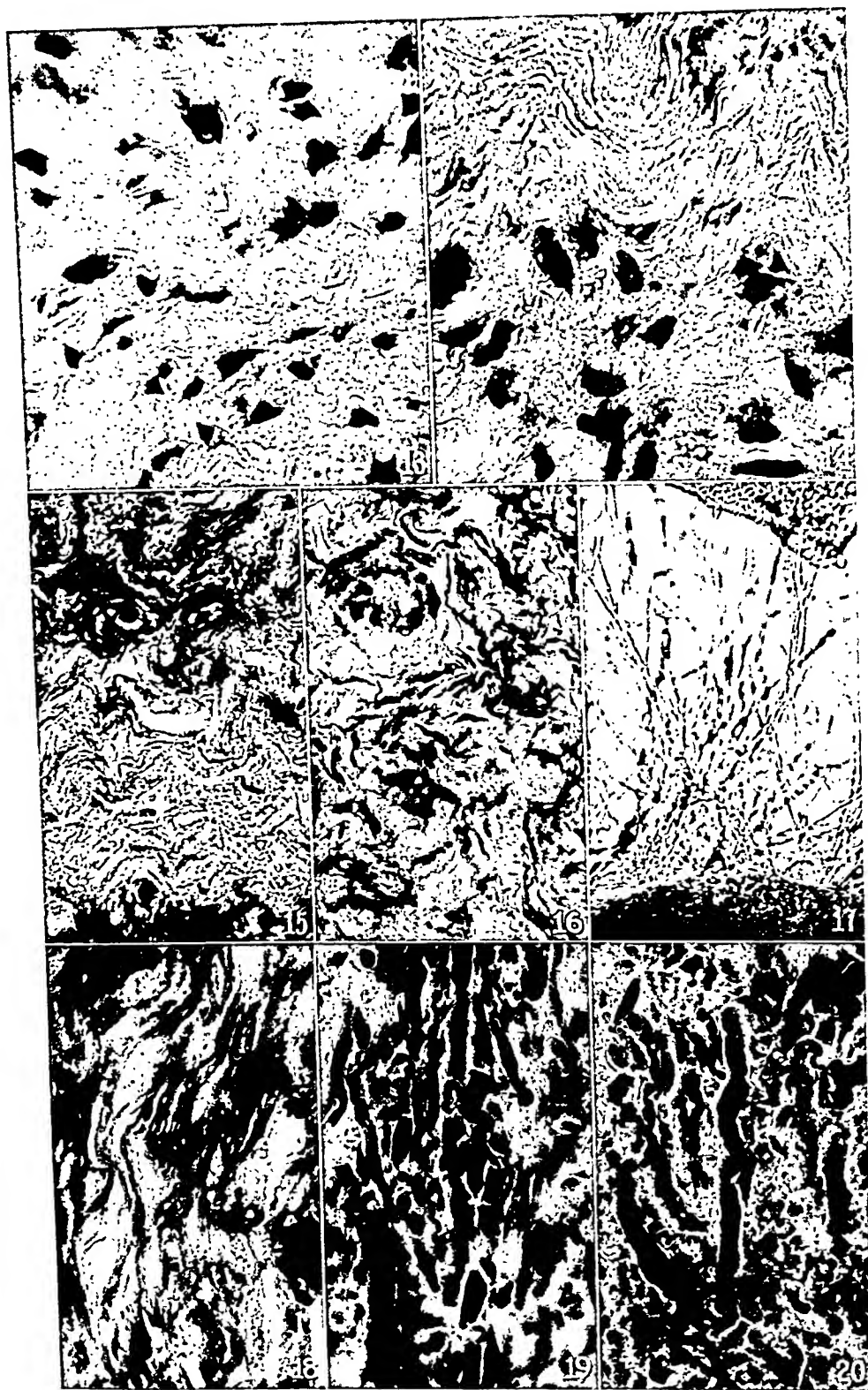
FIG. 18. Culture 6490-1. A section through the center of one of the original tissue fragments shown in Fig. 17. $\times 620$.

FIG. 19. Culture 6490-1. A section through the center of another fragment derived from the culture shown in Figs. 17 and 18. $\times 465$.

FIG. 20. Culture 6490-1. Another section through the center of the fragment shown in Fig. 19. $\times 465$.







(Parker: Cultivation of tissues in single flasks)

THE FATE OF A VIRULENT HEMOLYTIC STREPTOCOCCUS INJECTED INTO THE SKIN OF NORMAL AND IMMUNIZED RABBITS

By D. MURRAY ANGEVINE, M.D.

(From the Department of Pathology, Cornell University Medical College, New York)

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The fate of avirulent hemolytic streptococci after inoculation into the skin of normal and sensitized rabbits has been previously (1) described. When small numbers of relatively avirulent hemolytic streptococci were injected into the skin over the flank of normal rabbits they seldom diminished and usually increased in number during the first 5 hours after inoculation, but after 12 hours had usually disappeared. Streptococci reached the inguinal lymph nodes of normal animals in considerable number within 15 minutes; the number recovered was greatest within 2 hours after injection and gradually decreased so that few organisms were recovered from the lymph nodes after 7 hours. A smaller number reached the iliac lymph node. They were recovered in several instances from the blood and were occasionally found in the spleen.

When the same dosage of avirulent streptococci was injected into the skin of rabbits sensitized by repeated injection of avirulent hemolytic streptococci, the reaction at the site of inoculation was larger, more edematous and associated with more necrosis than in the normal animals. The avirulent streptococci multiplied for about 5 hours and were recovered in larger numbers from the sensitized than from the control animals. They persisted for a longer time in the skin of the sensitized (24 hours) than in that of the control rabbits. Streptococci were recovered from the inguinal lymph node in fewer instances and in much smaller numbers than from those of the controls. On no occasion did we recover streptococci from the iliac lymph node, blood, liver or spleen of sensitized animals. The multiplication in the skin of sensitized rabbits was favored by local injury with necrosis which

was more extensive than in the normal animals. In association with this more intense inflammatory reaction in the sensitized rabbits, the streptococci were fixed or held at the site of inoculation. It is evidently desirable to repeat the experiments with a hemolytic streptococcus virulent for rabbits.

The term sensitized was used in the above experiments because of the striking skin hypersensitivity produced by an avirulent hemolytic streptococcus which we have designated as strain B₁. It will be seen that the virulent hemolytic streptococcus, that will be designated as strain H, produces a very different picture. There is at first a moderate amount of cutaneous hypersensitiveness that gradually diminishes with subsequent injections of the organism. A very conspicuous degree of immunity indicated by diminishing local reaction is later produced. It seems appropriate in the following experiments to describe our animals as immunized against the virulent streptococcus.

Methods

Culture.—The virulent organism used in these experiments was a hemolytic streptococcus kindly given to us by Dr. F. P. Gay. It was originally isolated from a case of human empyema and has been used rather extensively by him during the past 15 years (2). He has designated it as *Streptococcus pyogenes* "H." The culture when received was in pleural fluid from a rabbit that had died with empyema. This pleural fluid was kept in the ice box, and cultures from it were designated as stock cultures. Subcultures were obtained by transferring 0.1 cc. of the pleural fluid to plain broth with a pH of 7.6 and incubating for 18 hours at 37°C. At first 1 cc. of a 1:100 dilution was fatal 7 days after intrapleural inoculation; this dosage was never fatal when injected intracutaneously. To increase the virulence of the organism, we followed successfully the method suggested by Gay (2). The stock culture was passed through the pleural cavity of several normal rabbits until 1 cc. of a 1:1,000,000 dilution proved fatal. Pleural fluid thus obtained was kept on ice and denoted the passage culture. In order to maintain a high virulence, the organism was passed through the pleural cavity of a normal rabbit once every 2 to 3 weeks. The virulence varied slightly from time to time, but was maintained at such a level that during the course of these experiments 1 cc. of a 1:10,000 dilution regularly caused the death of animals within 3 to 4 days. It is noteworthy that only one strain of streptococcus was used in these experiments.

Immunization.—Chinchilla rabbits ranging in weight from 1600 to 2400 gm. were used. They received intracutaneous injections of 0.1 cc. of 1:100 dilution

of an 18 hour stock broth culture of the H hemolytic streptococcus every week, usually for a period of 5 weeks. A few rabbits received one or two additional inoculations. In a few instances the initial dose was 0.1 cc. of a 1:10 dilution; however, this produced such large necrotic lesions that it was discontinued. The first injection produced uniformly large abscesses associated with much erythema, edema and a central yellow necrotic area. This lesion increased in size, usually for a period of from 4 to 5 days, and disappeared after from 5 to 6 weeks. In a few instances on the 5th or 6th day the erythema and edema surrounding the lesion spread for a considerable distance beyond it, resembling erysipelas, and then disappeared within 3 days.

The lesion produced by the second inoculation was usually slightly smaller than the first, there was less or occasionally no necrosis, and it usually disappeared in a somewhat shorter time. Subsequent inoculations regularly produced smaller red and edematous lesions with a somewhat firmer center; they reached a maximum size in 48 hours and rapidly subsided, disappearing entirely within from 3 to 4 days.

Test for Hypersensitivity.—Bottles (100 cc.) of broth with pH 7.6 were inoculated from the passage culture in pleural fluid and incubated for 48 hours at 37°C. The cultures were passed through a Seitz filter and the resulting filtrate tested for sterility. The filtrate was kept in the ice box and used as required. No deterioration was noted after 4 months in the ice box.

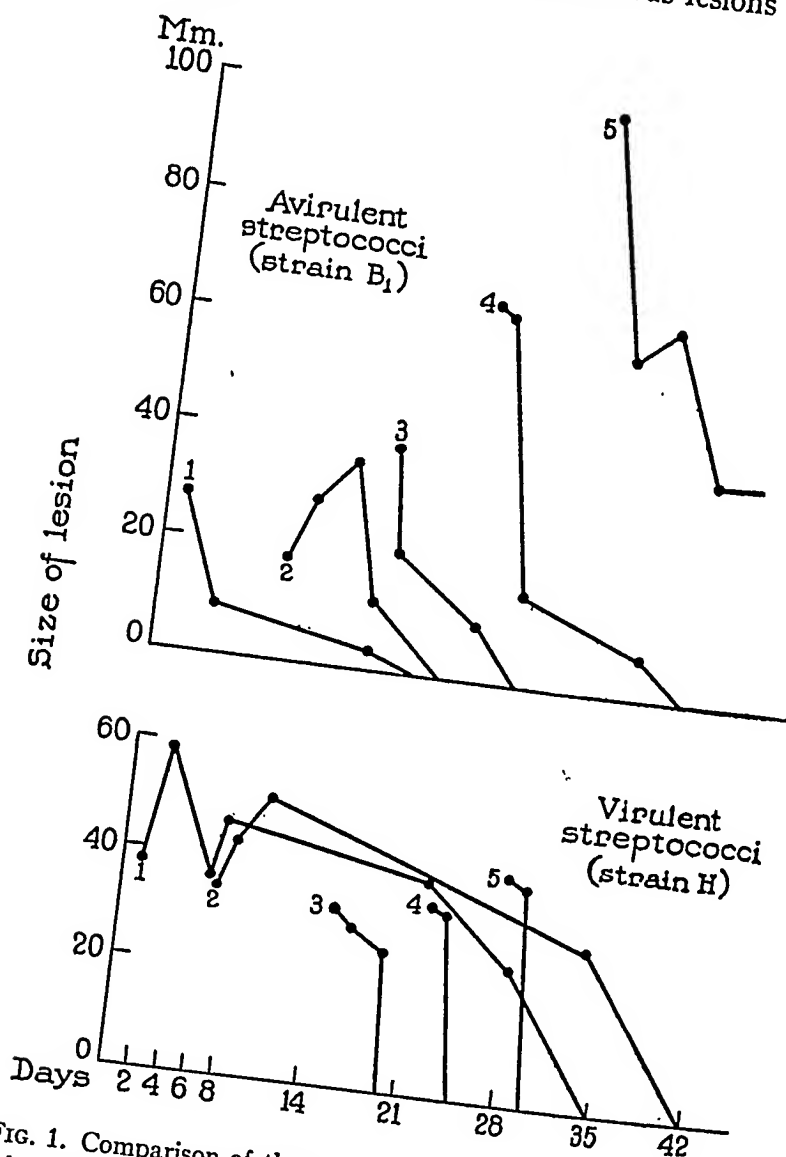
Skin hypersensitiveness to hemolytic streptococci was determined by an intracutaneous injection of 0.1 cc. of the streptococcus filtrate. Each rabbit was tested with filtrate before and at intervals during the period of immunization. The first injection was usually negative except for a slight diffuse erythema unaccompanied by edema in an occasional animal.

All immunized rabbits (Table I) developed some skin hypersensitivity to filtrate, although they usually reacted to filtrate more strongly about the 12th day than at the end of the period of immunization. The size of the edematous lesion produced with filtrate roughly paralleled that of lesions produced by simultaneous intracutaneous injections of living hemolytic streptococci.

A graphic comparison of the size and duration of the skin lesions produced by five injections of the virulent strain of hemolytic streptococci with those produced by the avirulent strain is given in Text-fig. 1. The two animals represent typical examples of similarly injected rabbits. With repeated injections of avirulent streptococci the lesions increase in size, becoming more erythematous and edematous; the later lesions also tend to persist longer. The first two injections of virulent streptococci, on the contrary, produce large ulcerative abscesses that heal after several weeks. Repeated injections usually

VIRULENT HEMOLYTIC STREPTOCOCCUS

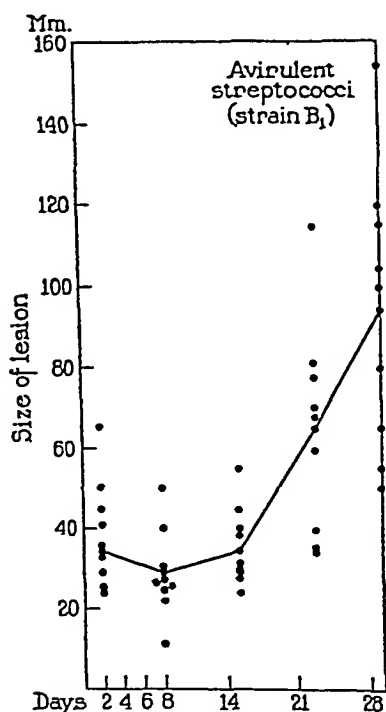
produce smaller erythematous and edematous lesions that disappear after 2 or 3 days.



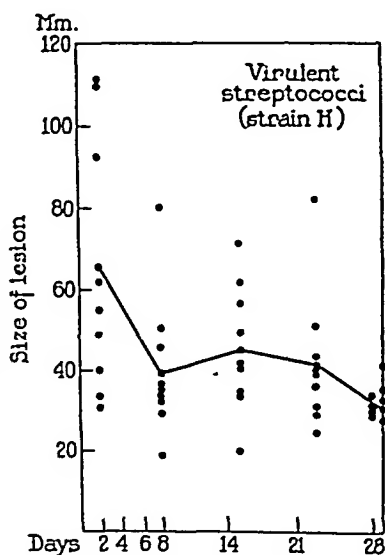
TEXT-FIG. 1. Comparison of the size and duration of lesions produced in the skin of rabbits by intradermal injections of 0.1 cc. of a 1:10 dilution of a broth culture of avirulent, and 0.1 cc. of a 1:100 dilution of virulent hemolytic streptococci. The size of the lesion represents the sum of the two longest diameters.

A comparison of the size of the lesions produced in nine comparable rabbits injected with avirulent and virulent hemolytic streptococci is given in Text-figs. 2 and 3. In the former instance, the increasing

size of the lesions is evident, whereas in the latter the lesions become much smaller and tend to be more uniform in size. The dosage of avirulent streptococci was 0.1 cc. of a 1:10 dilution, whereas that of the virulent streptococcus was smaller, being 0.1 cc. of a 1:100 dilution.



TEXT-FIG. 2



TEXT-FIG. 3

TEXT-FIG. 2. The size of lesions of nine rabbits, each receiving five subsequent injections of 0.1 cc. of a 1:10 dilution of a broth culture of avirulent hemolytic streptococci (strain B₁).

TEXT-FIG. 3. The size of lesions of nine rabbits, each receiving five subsequent injections of 0.1 cc. of a 1:100 dilution of a broth culture of virulent hemolytic streptococci (strain H).

It was desirable to use the smaller dose of virulent organisms so that extensive lesions might be avoided. A few animals were injected with the larger dose of 0.1 cc. of a 1:10 dilution of virulent hemolytic streptococci and a similar, but even more striking, degree of immunity was evident.

VIRULENT HEMOLYTIC STREPTOCOCCUS

EXPERIMENTS

In each experiment a normal and an immune rabbit of about the same weight received an intracutaneous injection of 0.1 cc. of a 1:100 dilution of an 18 hour passage culture of highly virulent H hemolytic streptococci on the left flank about

TABLE I
The Number of Streptococci Recovered from Normal and Immunized Rabbits after Intracutaneous Injection of the Organisms

Experiment No.	Time interval	No. of organisms injected 0.1 cc. 1:100	No. of organisms recovered							
			Normal rabbits				Immune rabbits			
			Skin	Lymph node		Blood	Spleen	Skin	Lymph node	
				Inguinal	Iliac				Inguinal	Iliac
	min.									
1	15	130,000	201,000	0	—	0	0	61,000	0	—
2	1	290,000	2,320,000	Few	0	0	0	0	0	0
3	2	350,000	2,221,000	4180	144	0	0	1,023,000	Few	0
4	4	160,000	10,800,000	75	0	0	0	1,720,000	70	0
5	6	130,000	26,800,000	2700	4	0	0	560,000	0	0
6	8	190,000	121,000,000	5550	16	Pos.	50	17,000	0	0
7	12	290,000	150,000,000	11,450	0	0	20	6,600,000	400	0
8	21	350,000	23,200,000	1535	0	0	0	2200	0	0
9	24	290,000	7,530,000	714	—	0	0	0	0	0
10	2	600,000	43,000,000	30,340	1072	Pos.	300	190,000	556	—
11	3	600,000	1,350,000	4600	0	0	0	5000	9	0
12	4	190,000	8,600,000	3200	1000	0	100	0	0	0
13	5	130,000	3,610,000	43,950	52	Pos.	30	0	0	0
14	7	130,000	230,000	360	0	0	0	0	0	0
15	9	130,000	1,870,000	0	0	0	0	0	0	0
16	13	190,000	93,000	0	0	0	0	0	0	0
17	16	200,000	1,950,000	0	0	0	0	0	0	0
18	19	200,000	0	0	0	0	0	0	0	0
19	21	200,000	5000	0	0	0	0	0	0	0

4 cm. cephalad to the left ilio-inguinal lymph node. The number of organisms injected was estimated by the procedure used in the former study (1). Lesions at the site of inoculation in the control rabbits, evident after 4 hours, were erythematous and measured about 11 by 12 by 1 mm. They increased

during 48 hours, when they appeared as large, edematous, ulcerated lesions of which the largest measured 51 by 37 by 9 mm.

In the immunized rabbits pale, edematous, occasionally pink wheals appeared within 4 hours. The inflamed area did not increase in size; however, in some instances after 48 hours, it measured 25 by 25 by 2 mm., and had a small yellow center. In no instance did ulceration occur.

The skin lesions, inguinal lymph nodes, iliac lymph nodes and spleens were removed and cultured as previously described (1). A culture from the blood of the heart was made immediately before death from each rabbit. The number of organisms injected and the number recovered are given in Table I. We made no cultures from the immune animals after 4 days because no organisms had been recovered from this group from 2 to 4 days after inoculation.

The number of organisms injected varied from 130,000 to 600,000, with an average of 300,000 organisms for each injection.

Number of Streptococci at the Site of Injection.—The organism was recovered in large numbers from the normal animals in every instance. In the first twelve experiments, from 201,000 to 150,000,000 organisms (average about 35 million) were recovered from the skin of the normal rabbits, as contrasted with from 0 to 6,600,000 organisms (average about 800,000) from the twelve immunized rabbits. In normal rabbits multiplication began during the first 2 hours, proceeded rapidly for 12 hours and then diminished slightly up to 24 hours. Large numbers of streptococci were recovered from both inguinal and iliac lymph nodes, blood and spleen, during 5 days after injection, but none were recovered after the 7th day because the rabbits had become immune. Two of three animals injected at the same time, not included in Table I, died with bacteremia on the 7th day.

Streptococci were recovered from rabbits killed from 5 to 16 days after injection. On the 19th day the site of injection was found to be sterile, and a few organisms were recovered from a normal rabbit examined on the 21st day. Irregularities in the numbers recovered from normal rabbits may be due in part to individual differences in rabbits; it is probable that in some instances a great many organisms were lost in purulent discharge, whereas in others there was little or none.

The number of organisms recovered from the skin of the immune rabbits was less than the number injected in all save 4 instances, and in these there was some multiplication. The greatest multiplication

VIRULENT HEMOLYTIC STREPTOCOCCUS

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2	1 hrs.	290,000	2,320,000	Few	0	0	0	1,023,000	Few	0
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4	4	160,000	10,800,000	75	0	0	0	560,000	0	0
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15	9	130,000	1,870,000	0	0	0	0	0	0	0
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The number of organisms recovered from the skin of the immune rabbits was less than the number injected in all save 4 instances, and in these there was some multiplication. The greatest multiplication

VIRULENT HEMOLYTIC STREPTOCOCCUS

occurred in an animal killed 8 hours after injection (Table I); however, 121 million, or 18 times as many organisms, were recovered from the corresponding control rabbit. In one immune rabbit the organisms had entirely disappeared from the site of injection within 21 hours and none were recovered after 48 hours. Variations in the numbers recovered probably bear some relationship to variations in the degree of immunization produced in animals.

Inguinal Lymph Node.—There are conspicuous differences in the number of streptococci in the inguinal lymph nodes of the two groups (Tables I and II). In the first twelve experiments, an average of 5362 organisms was recovered from the inguinal lymph nodes of the normal,

TABLE II
Number of Normal and Immunized Rabbits with Streptococci in Lymph Nodes, Blood and Spleen

Culture taken from	Normal rabbits			Immunized rabbits		
	No. of rabbits	Showing hemolytic streptococci in culture		No. of rabbits	Showing hemolytic streptococci in culture	
		No.	per cent		No.	per cent
Inguinal lymph node	12	11*		12	6†	
Iliac lymph node	10	5	91.6	10	0	50
Blood	12	2	50	12	0	—
Spleen	12	4	16.6	12	0	—
			33			—

* Streptococci averaged 5362 per lymph node.

† Streptococci averaged 86 per lymph node.

as contrasted with an average of 86 organisms in those of the immune rabbits. The number of organisms in the lymph nodes appears to be associated in a general way with multiplication at the site of inoculation and the greater the multiplication in the injected skin, the greater is the passage of organisms to the adjacent lymph node. Streptococci were recovered in relatively large numbers from all except one of twelve inguinal lymph nodes of normal rabbits. The inguinal lymph node removed after 15 minutes was sterile. Streptococci were recovered in smaller numbers and from only six of twelve inguinal lymph nodes of the immune rabbits.

Iliac Lymph Node.—The iliac lymph nodes were not found with

certainty in two instances and hence they were omitted from the enumeration in Tables I and II. We were able to culture an average of about 223 colonies of hemolytic streptococci per lymph node from five of ten normal rabbits. The other five were sterile. Streptococci were usually recovered from the iliac nodes when large numbers of organisms were present in the inguinal lymph node. The iliac lymph nodes of immune rabbits gave no growth.

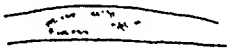
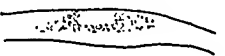
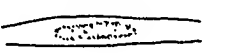
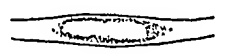
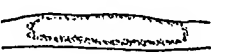
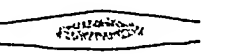
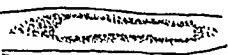
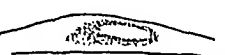
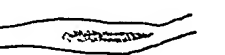
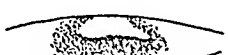
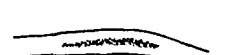

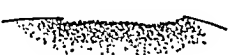
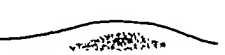
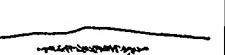
Blood.—Positive blood cultures were obtained from two of twelve normal rabbits killed within 4 days after inoculation and in these there was a great deal of multiplication of streptococci in the skin; they were numerous in the inguinal lymph nodes and were present in the iliac lymph nodes. Positive cultures were obtained from the blood only in animals with streptococci in the iliac lymph nodes. Cultures made of the blood of immune animals were in all instances sterile.

Spleen.—Organisms were grown from the spleen in four of the twelve normal animals killed within 4 days after inoculation. In all instances in which streptococci were found in the spleen, they had undergone conspicuous multiplication at the site of inoculation, were numerous in the inguinal lymph nodes and were recovered from iliac nodes. The spleen from immune rabbits was sterile in each instance.

Histological Changes at the Site of Injection and in Adjacent Lymph Nodes in Normal and Immunized Rabbits

A group of six rabbits received five intracutaneous injections of 0.1 cc. of a 1:100 dilution of living virulent hemolytic streptococci at weekly intervals. After the third injection of streptococci a test dose of filtrate produced edematous wheals. The average lesion for the entire group was about 22 mm. across and 2 mm. high. After the fifth injection, although there was some hypersensitivity to the filtrate, the reaction was considerably less. It was only slightly elevated above the skin surface and measured 12 cm. across. This group together with six normal rabbits received an intradermal injection of 0.1 cc. of a 1:100 dilution of an 18 hour broth culture of virulent streptococci (190,000). The site of injection was carefully marked with India ink and this remained visible until the sections were cut. An immunized and a control rabbit were killed simultaneously after intervals of 2, 4, 8, 12, 24 and 48 hours. The skin at the site of injection

tion and the inguinal lymph nodes were placed in Zenker's fixative, sectioned and stained with Mallory's eosin and methylene blue. Text-fig. 4 gives diagrammatic cross sections of the skin lesions. The dark stippled area represents the extent of cellular reaction, and the clear central area the site at which the streptococci are seen. Descriptions of the skin lesions in two groups of animals follow.

Hours after injection	Skin lesions with hemolytic streptococci (0.001 cc.)		
	Virulent (strain H)		Avirulent (strain B ₁)
	Normal	Immunized	Normal
4			
8			
12			
24			
48			

TEXT-FIG. 4. Diagrammatic representation of the skin lesions (enlarged 1.5X) in normal and immunized rabbits. The lesions were removed at intervals after intracutaneous injections of 0.1 cc. of a 1:100 dilution of a broth culture of virulent hemolytic streptococci (strain H). Lesions from normal rabbits that received the same dose of avirulent hemolytic streptococci (strain B₁) are included for comparison.

After 2 Hours.—

R 1, Control.—The site of inoculation is not visible. There is infiltration of polymorphonuclear leucocytes throughout the corium, together with a few lymphocytes and mononuclear cells. A few cocci are seen to be free between the collagen fibres and in very few instances one is found within the cytoplasm of a polymorphonuclear leucocyte.

R 2, Immunized.—There is no alteration in the skin that is visible to the naked eye. Microscopical changes are similar to those described above but there are fewer polymorphonuclears. A considerable number of them contain blue staining cocci. The organisms are recognized with much difficulty in intact cells, because they are obscured by the eosinophilic granules. On the contrary, in damaged cells with few or no granules they are recognized readily.

After 4 Hours.—

R 3, Control.—There was some edema and faint erythema at the site of inoculation. Many polymorphonuclear leucocytes are scattered throughout the section and some are in foci. A moderate number contain cocci singly and in pairs. Extracellular streptococci are readily discernible in considerable numbers.

R 4, Immunized.—The skin was slightly injected. Microscopically there are large numbers of streptococci occupying an area containing few cells still intact. This area is surrounded by a fairly extensive accumulation of polymorphonuclear leucocytes, a few lymphocytes and mononuclear leucocytes. A moderate number of polymorphonuclears adjacent to the streptococci show phagocytosed organisms. They are seen with difficulty in the cells with numerous granules and in areas where there is marked fragmentation and pyknosis of the nuclei.

After 8 Hours.—

R 5, Control.—The skin lesion measures 12 by 11 by 1 mm. and is intensely hemorrhagic with a central dark purple area. A red streak upon the skin corresponds to the lymphatic draining this area. There are large numbers of organisms in the section and an extensive infiltration of polymorphonuclear leucocytes. Streptococci are not limited to the site where polymorphonuclear leucocytes occur but are seen in considerable numbers in the tissue beyond the barrier of leucocytes and here they have apparently multiplied. A moderate number of polymorphonuclear cells contain single cocci.

R 6, Immunized.—There is a pale edematous wheal on the skin surface that measures 12 by 18 by 2 mm. A large number of streptococci, but definitely fewer than in the control animal, are scattered between the collagen fibrils within a dense zone of polymorphonuclear leucocytes that serves as a barrier. Cells adjacent to organisms are largely disintegrated whereas those at the periphery are intact. At the junction of well preserved polymorphonuclears and those that have lost their granules there is a considerable amount of phagocytosis.

After 12 Hours.—

R 7, Control.—The skin lesion measures 20 by 14 by 3 mm. with a yellow necrotic area in the central part. Streptococci are present in larger numbers than in any previous section. They occupy a large area with few cells in which the areolar tissue is edematous; this area is palely stained in contrast to the surrounding dense, fairly complete zone of polymorphonuclear leucocytes. There is a moderate amount of phagocytosis and cells that have ingested streptococci often have damaged nuclei and diminished granules.

R 8, Immunized.—The skin lesion measures 12 by 12 by 2 mm. with a very small yellow center (two other immunized rabbits, R 10 and 12, show no lesion at the site of inoculation at this time). A very dense collar of polymorphonuclear leucocytes surrounds a small clear area with few cells and relatively large numbers of streptococci. The nuclei of the innermost cells are greatly fragmented. The intact polymorphonuclear leucocytes show considerably more phagocytosis than do those in the control rabbit.

After 24 Hours.—

R 9, Control.—A superficial abscess is present on the skin; it measures 13 by 18 by 2 mm., with a definite yellow central part. There is extensive necrosis of the areolar tissue of the corium. Practically no cells but great numbers of streptococci in chains and clumps are present in this area. The necrotic tissue is surrounded by a wide dense zone of polymorphonuclear and mononuclear leucocytes and lymphocytes. A slight amount of phagocytosis is noted.

R 10, Immunized.—The site of injection shows slight redness that measures 6 by 6 mm.; there is no edema. In the section there is a dense collection of polymorphonuclear leucocytes associated with localized necrosis of tissue. There are many mononuclear macrophages, some of which have ingested polymorphonuclear leucocytes. Very few cocci are seen free in the tissue. A considerable number are contained within the cytoplasm of both the polymorphonuclears and macrophages.

After 48 Hours.—

R 11, Control.—The skin lesion is a large abscess that measures 35 by 25 by 4 mm.; superficial ulceration measures 1 cm. across. A section shows that the abscess involves all layers of the skin; the central part is necrotic. There are many polymorphonuclear and mononuclear leucocytes throughout the edematous tissue. A few small clusters of blue-staining cocci are seen free in the tissue. A relatively large number are contained within the polymorphonuclear leucocytes.

R 12, Immunized.—The skin lesion is quite erythematous and measures 12 by 12 by 2 mm. There is no gross evidence of necrosis. The section of skin contains a well localized abscess composed of necrotic cells and tissue surrounded by a zone of polymorphonuclear leucocytes, together with a large number of macrophages that have ingested polymorphonuclear leucocytes and contain a considerable amount of nuclear debris. A few scattered cocci are present, most of them within the cells.

Inguinal Lymph Nodes

The changes in the lymph nodes parallel rather closely those of the corresponding skin lesions described above. The lymph nodes from the normal and immune rabbits do not differ conspicuously either in the gross or microscopically. However, several features are worthy of note.

Control Rabbits.—In the normal rabbits the lesion is associated with a greater polymorphonuclear infiltration up to 12 hours. Mononuclear leucocytes appear in fairly large numbers after 12 hours and a few scattered cocci are seen in practically every section. Within 24 hours small focal abscesses, quite definite after 48 hours, are appearing; after 4 days these abscesses become confluent. At this time suppurating thrombi occlude many of the lymphatics on the periphery of the node. After 7 days in an animal dying with a generalized bacteremia there are

somewhat similar changes, but mononuclear cells have entered these lymphatics and there are large clumps of bacteria in necrotic lymphatic tissue and in the cortical lymph sinus.

Immune Rabbits.—In the immunized rabbits inflammation is somewhat less intense and at 48 hours is definitely subsiding with a diminution in number of polymorphonuclears. The mononuclear leucocytes appear after 4 hours in moderately large numbers. It is difficult to compare the number of microorganisms seen in the two groups; however, in several of the immune animals they were found with more difficulty and were probably present in smaller numbers.

From observations described above, it is evident that in normal rabbits streptococci multiply at the site of inoculation in most instances, and rapidly cause the formation of a large local abscess. When the rabbit recovers the lesion heals and the organisms disappear, whereas if the animal dies with bacteremia streptococci are found in large numbers at the site of inoculation as well as in the adjacent lymph nodes. No deposition of fibrin was seen in the skin lesions; however, after 4 days many of the lymphatics are occluded with suppurating thrombi. In the immune group the site of injection remains smaller, never ulcerates, and the inflammation disappears within a relatively short time. There is more phagocytosis in the lesions of the immune than in those of the normal rabbits.

Comparable skin sections from rabbits injected with an avirulent hemolytic streptococcus are included in Text-fig. 4. It is readily seen that rabbits immunized with a virulent streptococcus have reacted to subsequent injections of the same organism in much the same way that normal animals have reacted to a first injection of an avirulent streptococcus.

DISCUSSION

In comparing the fate of virulent hemolytic streptococci injected into both normal and immunized rabbits with that of avirulent streptococci under the same conditions, only one strain of each has been used. Smaller doses of virulent than of avirulent streptococci have been used in most instances to produce immunization.

When small amounts (0.1 cc. of a 1:100 dilution) of virulent hemolytic streptococci were injected into the skin of normal rabbits they multiplied at the site of inoculation and were still present after 3 weeks (Table I). The organisms invaded the lymph nodes and were demon-

VIRULENT HEMOLYTIC STREPTOCOCCUS

strable in large numbers up to 7 days. In several animals, they were recovered from the blood stream and spleen. Two animals died. When avirulent hemolytic streptococci were injected intracutaneously the result was different. The organisms multiplied for a short time in the skin and were practically all dead after 12 hours. They passed to the lymph nodes in small numbers for a short time after inoculation and a few were obtained from the heart's blood and spleen. When larger doses were given organisms were not recovered from the blood as with the more virulent organisms.

Although there was some slight multiplication of avirulent hemolytic streptococci in the skin of normal rabbits after intracutaneous injection (1), it never at any time approached the degree of multiplication of virulent hemolytic streptococci injected into the skin of normal rabbits. The numbers of streptococci recovered from the inguinal lymph nodes were also much greater following the injection of virulent organisms.

There are few observations on the fate of bacteria injected into the skin but numerous studies of inoculation into the blood stream (Buxton and Torrey (3), Bull (4)). Hopkins and Parker (5) found that a sublethal dose of hemolytic streptococci with a low virulence for rabbits disappeared from the blood stream in a few hours. After a lethal dose (5) over 90 per cent of the cocci were removed from the blood stream in the first few minutes; they were at a minimum at 2 to 3 hours, but after 4 to 6 hours they began to increase and persisted until the death of the animal. In our experiments, after intracutaneous injection there is seldom if ever any immediate local diminution, but multiplication begins during the first 2 hours.

Many workers have been able to immunize rabbits against streptococci, whereas others have been less successful.

Gay and Rhodes (6), using living cultures of the H streptococcus that Dr. Gay kindly furnished to us, were able to protect rabbits against experimental erysipelas produced by intradermal injections of the same strain of hemolytic streptococci. McLeod (7) on the other hand experienced great difficulty in immunizing rabbits against strains of streptococci of which the virulence may be enhanced by animal passage. Varying results have been obtained with heat-killed vaccines. Hopkins and Parker (5) were unable to protect rabbits against streptococci with repeated intravenous inoculations of heat-killed organisms over a period of 8 months. Day (8) immunized white mice with intraperitoneal injections of heat-killed

vaccines prepared from both fully virulent and less virulent streptococci of the same strain. The mice then received intraperitoneal injections of living virulent streptococci. It was found that the mice immunized with the vaccine prepared from the fully virulent streptococcus were better protected than those immunized with the vaccine prepared from the less virulent streptococcus. He concluded that unless the virulence was of a high degree vaccines failed to give protection even to streptococci of submaximal virulence and might even induce susceptibility.

Downie (9) attempted to immunize rabbits with the toxin prepared from filtrates and with heat-killed cocci of a moderately virulent (Dochez) strain and with a highly virulent (Davis) strain. He found that the toxin of the moderately virulent strain immunized against a fatal infection after intradermal or intravenous injection of the same strain, whereas the coccus-immunized rabbits and the controls died. The filtrate of a highly virulent organism afforded little protection when given either intradermally or intravenously against an homologous strain, whereas the heat-killed cocci protected against an homologous strain (Davis) but not against a less virulent (Dochez) one. This group of experiments demonstrates some of the difficulties in the interpretation of streptococcus immunity. Those who have immunized with the living organisms have had more success.

The experiments that have been described show that rabbits inoculated with a moderately virulent H streptococcus are well immunized against the same strain of streptococcus even after the virulence has been considerably increased by animal passage. Injections controlled subsequent inoculations of streptococci with increasing efficiency, demonstrated by both gross and microscopic changes and by bacteriological examinations (Tables I and II). These animals were not tested with increasing doses of streptococci to determine the degree of acquired immunity, and observations concerning immunity are on a qualitative rather than a quantitative basis. The observation of this conspicuous immunity produced in association with a diminishing and slight degree of hypersensitivity, is one of many observations of a high degree of immunity with scant if any hypersensitiveness. The rabbits immunized with virulent streptococci did, however, have some cutaneous hypersensitivity to the streptococcus filtrate, though this was at a maximum before the end of immunization, so that these experiments give no information concerning the relationship between hypersensitivity and immunity.

The histological sections of lesions from normal and immunized rabbits show more phagocytosis in the immune than in the normal rabbits. The early experiments of Denys and Leclef (10) on the

phagocytosis of streptococci showed that there was more phagocytosis by the polymorphonuclear leucocytes in vaccinated rabbits than in normal rabbits. Corresponding observations were made *in vitro*.

It is noteworthy that skin lesions following the injection of avirulent hemolytic streptococci into normal rabbits (1) are similar in gross and histological characters to lesions produced by a virulent hemolytic streptococcus in rabbits that had received repeated injections of virulent streptococci. In other words, a virulent hemolytic streptococcus produced much the same type of reaction in immunized rabbits that an avirulent streptococcus produced in normal rabbits. The fate of the streptococci determined by counting of colonies was found to be similar in the two instances.

The relationship of virulence to the production of immunity is still obscure, although there are several observations indicating that increased virulence promotes immunization. The ability of virulent hemolytic streptococci to multiply in the body and invade the blood stream may help to explain why animals injected with virulent organisms develop a high degree of immunity.

SUMMARY AND CONCLUSIONS

1. Hemolytic streptococci, highly virulent for rabbits, when injected into the skin of normal animals increased greatly in number at the site of injection during the first 12 hours, diminished somewhat after 24 hours but still persisted after 21 days. They produced large abscesses within 24 hours, there was ulceration, and healing occurred after about 5 weeks. Histological observations confirmed the bacteriological evidence that the streptococci underwent great multiplication at the site of their injection.

2. Virulent hemolytic streptococci injected into the skin of normal rabbits appeared in small numbers within 1 hour in the lymph nodes. As multiplication proceeded in the skin, hemolytic streptococci were found in large numbers from 1 hour to 7 days after inoculation in the inguinal lymph nodes.

3. Hemolytic streptococci were recovered from deeper lymph nodes, that is, from the iliac nodes, but only in animals of which the inguinal lymph nodes contained bacteria in relatively large numbers.

4. Virulent hemolytic streptococci injected into the skin of normal

rabbits in some instances entered the blood stream in considerable number, and occasionally caused death with bacteremia. Streptococci were recovered more frequently from the spleen and were present in this organ only when they had been recovered from the deep (iliac) lymph nodes.

5. When virulent hemolytic streptococci were injected into the skin of immunized rabbits, in a few instances they increased in number for a short time, but usually diminished rapidly and had entirely disappeared in 48 hours. The gross lesions were smaller than in normal rabbits. There was more phagocytosis, and redness and edema had disappeared after 48 hours.

6. When virulent streptococci were injected into the skin of immune rabbits they passed to the regional lymph nodes in relatively smaller numbers than in the previously normal controls and appeared in these nodes in considerable numbers only in animals in which there had been conspicuous multiplication at the site of inoculation. No streptococci could be found in the iliac lymph node, blood or spleen.

7. Virulent streptococci injected into the skin of normal animals multiply actively, resist phagocytosis, invade the tissues widely, enter adjacent and distant lymph nodes and in some instances are distributed by the blood stream to internal organs. After immunization associated with some sensitization, virulent streptococci are more readily ingested by phagocytes, remain sharply localized, are rapidly destroyed, fail to pass the nearest lymph nodes and do not enter the blood stream.

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THE IMMUNOLOGICAL SPECIFICITY OF STAPHYLOCOCCI

IV. CUTANEOUS REACTIONS TO THE TYPE-SPECIFIC CARBOHYDRATES*

BY L. A. JULIANELLE, PH.D., AND A. F. HARTMANN, M.D.

(From the Oscar Johnson Institute and Children's Hospital, Washington University School of Medicine, St. Louis)

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Previous communications from this laboratory have described the occurrence of two immunological types of *Staphylococcus* which are discernible by the precipitation in homologous immune sera of specific carbohydrates extracted from the organisms (1). A study of the chemical characteristics of the carbohydrates revealed sufficient differences to explain the serological distinctions of the two substances (2). In pointing out the essential immunological dissimilarities of the carbohydrate and protein constituents of the cells, a preliminary statement was made, among other things, regarding the cutaneous reactions to the specific polysaccharides (3, 4). Since that time, however, a more thorough and broader study has been undertaken on the skin reactions elicited by the carbohydrates in both normal and diseased individuals, with the result that sufficient data are now available to merit an extended report and conclusions.

Materials and Methods

1. *Soluble Specific Substances.*—The specific carbohydrates of both Type A and Type B *Staphylococcus* were employed in conducting skin tests, and they were prepared according to the method already published from this laboratory (2). While in a high state of purity, liberated from the usual contaminating and protein substances, they still contained small amounts of nitrogen (3 to 4 per cent), but, as will be seen later, this degree of impurity exerted no visible influence on their skin reactive properties. For purposes of control, use was made of the specific substance of Type II *Pneumococcus*, prepared by the method of Heidelberger, Goebel and Avery (5), the serum-reactive carbohydrate of gum arabic (6), and the somatic polysaccharide of a rough strain of *rhinoscleroma bacillus* (7),

* Conducted under a grant from the Commonwealth Fund of New York.

which was prepared in a manner similar to that used for the extraction of carbohydrates from *Staphylococcus*. The various substances were dissolved in freshly prepared physiological salt solution and were sterilized in a boiling water bath for 10 minutes. The concentration of the carbohydrates was varied from 1:50,000 to 1:200,000, and 0.2 cc. of the particular dilution was injected intracutaneously for testing skin reactivity. At the present time, the dilution and quantity of carbohydrate injected for skin testing has been standardized at 0.2 cc. of 1:200,000 dilution.

2. *Titration of Sera for Specific Precipitins*.—Serum was obtained from normal individuals and patients in the usual manner. Precipitation tests were carried out by mixing 0.5 cc. of different dilutions of carbohydrate with 0.5 cc. of diluted serum (i.e., 0.2 cc. of serum and 0.3 cc. of salt solution). Readings were made after the tests had been incubated in a water bath at 37°C. for 2 hours followed by overnight in the ice box.

3. *Titration of Urine for Specific Carbohydrates*.—In the patients sustaining severe infection with *Staphylococcus*, an effort was made to demonstrate the presence of specific carbohydrates in the urine. For this purpose 24 hour specimens of urine were collected from time to time and aliquot portions were removed for testing. If necessary, they were then centrifugated for clearing, and 0.5 cc. of urine, undiluted and in dilutions varying from 1:1 to 1:200, was mixed with 0.5 cc. immune rabbit serum (diluted 2:3). Then the test was completed as described in the preceding paragraph.

EXPERIMENTAL

For purposes of clearer presentation and more facile discussion, the cutaneous reactions to the carbohydrates of *Staphylococcus* have been divided as those observed in infants and children, and those observed in adults. While the character and evolution of the reaction is the same in both groups, there are, nevertheless, differences in other respects which warrant this separation. In the former group individuals varied in age from a few months to about 8 years, and the adults varied from adolescence to beyond middle age. In both instances, a survey was made of normal individuals, as well as of patients suffering from various diseases, supplemented by patients with verified *Staphylococcus* infection.

The skin reaction elicited by the carbohydrates is of the immediate, wheal and erythema variety.

Within 10 minutes following the inoculation a white edematous wheal appears at the site of inoculation, which, in a general way, varies in diameter from 0.75 to 1.5 cm., although in occasional instances the

measurement may attain 2.0 or more cm. Within 10 to 20 minutes, a zone of erythema appears surrounding the wheal. The erythema may increase in size and intensity, frequently exhibiting an irregular margin and extending unevenly in the form of pseudopods. Within 30 minutes, the reaction usually assumes maximum intensity, when it begins to regress, in general completely vanishing within an hour, sometimes requiring a longer period. The eventual size of the reaction varies in different individuals. Considering the wheal and erythema in one measurement, the two diameters show variations of 1×1.5 cm. to 4×6 cm. The skin reaction has not been followed by secondary, local or constitutional manifestations.

This cutaneous reaction will be recognized immediately as that described for the first time in response to bacterial carbohydrates by Tillett and Francis in their study of skin reactions in *Pneumococcus pneumonia* (8). An observation of further interest was the occurrence of a prozone phenomenon in the skin reactivity of some of the individuals, which may be likened to that occurring in serum precipitation reactions. For example, dilutions of 1:20,000 or 1:50,000 failed to elicit skin reactions, though 1:100,000 or 1:200,000 did so.

Cutaneous Reactions in Children

The skin tests performed in children have been summarized in Table I. An analysis of the data discloses that of 40 children with miscellaneous clinical conditions, five, or about 12 per cent, gave skin reactions to the carbohydrate of Type A *Staphylococcus*. Of this group, 20 were tested to Type B carbohydrate without exhibiting cutaneous response. These 40 children are considered as normal, control individuals for the purpose of this study. None of them gave evidence of *Staphylococcus* infection during their period of observation in the Children's Hospital, and most of them were patients classified as metabolic. A few were suffering from infections, as *e.g.* typhoid fever, actinomycosis, pyelitis, etc. It seems justifiable, therefore, to regard the children of this group as fair for control purposes. Sera from two of the five reactive children were tested for precipitins, and in neither instance were antibodies demonstrable for Type A or Type B carbohydrate.

In a group of 11 children with hemorrhagic nephritis, only one

reacted to Type A carbohydrate, and none to Type B. Precipitation tests performed with serum from two patients, one of whom reacted and the other of whom did not, revealed an absence of specific antibodies in circulation. These patients are classified separately because they serve as a control for the patients suffering from nephrosis.

Seven children with nephrosis, also, were skin-tested with the various carbohydrates. All of them were found reactive to Type A polysaccharide but not to Type B during the symptoms of active disease. None of the children's sera contained specific precipitins. The children have been studied over a period of 2 years, and skin tests have

TABLE I

Summary of Skin Tests and Serum Reactions in Children to Specific Carbohydrates of Staphylococcus

Individuals	Skin tests					Serum reactions		
	No. tested	No. reacting to carbohydrate		Per cent reacting to carbohydrate		No. tested	No. reacting with carbohydrate	
		Type A	Type B	Type A	Type B		Type A	Type B
Miscellaneous.....	40	5	20 tested all negative	12	—	2	0	0
Nephritis.....	11	1	0	9	—	2	0	0
Nephrosis.....	7	7	0	100	—	7	0	0
Staphylococcus infection..	10	10	0 1 questionable	100	—	10	5	0

been made repeatedly during and after hospitalization. They have presented two chief points of interest. First, it has been observed that during their prolonged hospitalization, the occurrence of upper respiratory infection may precede a disappearance of the skin reactivity to Type A carbohydrate. In this case, skin reactivity reappears with recovery from the intercurrent infection. This observation, although not conclusive, was made on several occasions, so that it would appear to be of more than fortuitous occurrence. Secondly, upon following the children clinically after discharge, the recurrence of skin reactivity is frequently accompanied by albuminuria, so that it may be the latter condition is predictable from the presence of skin reactivity.

Since the number of patients with nephrosis studied is, indeed, very small, it is difficult to interpret the significance of these observations. As will be brought out subsequently, the absence of circulating antibodies may or may not be important as far as specific infection is concerned. The occurrence of nephrosis, however, is rare, and a long period of time must elapse before sufficient numbers are available for a statistical study.

Three children with nephrosis have been tested with the carbohydrates at our request by Dr. J. H. McLeod, of Washington, D. C., who reported in two of the three patients a skin reactivity to Type A and not to Type B carbohydrate. Another patient, tested by Dr. Fred Moore, of Des Moines, Iowa, was found to be non-reactive. Four patients with nephrosis were tested by Dr. L. E. Farr, of the Hospital of The Rockefeller Institute; none gave skin reactions to Type A carbohydrate, while two gave borderline reactions to both Type B carbohydrate and the soluble specific substance of Type I Pneumococcus.

Finally, ten children with authenticated *Staphylococcus* infection were tested with the different carbohydrates. All ten gave typical reactions to Type A carbohydrate, while one reacted slightly to Type B. The latter reaction was considered of doubtful significance, since the patient showed a mild generalized skin reactivity and appears to have been dermatographic. Tests of serum from these patients indicated the presence of specific precipitins in five. The serological reactions will be described later, however.

Cutaneous Reactions in Adults

The skin tests performed on adults are presented in summary form in Table II. A study of this protocol shows that of 24 normal individuals, all medical students enjoying perfect health at the time, 16, or 66 per cent, were reactive to Type A carbohydrate. In two, less intense reactions were also observed to Type B carbohydrate, but these were classified as questionable because the individuals presented a minor degree of reactivity to all the polysaccharides inoculated. Subsequently, the sera of five giving reactions were tested for both Type A and Type B precipitins, but with a negative outcome.

Skin tests were also made on 47 patients with trachoma. In every case, staphylococci were cultivated from the conjunctival sac, but it is doubtful that this observation is of any importance. Type A carbo-

hydrate elicited reactions in 30 of the patients, while Type B carbohydrate caused a minor reaction in only two. Serum from 16 of the patients, including the two reacting questionably to type B, was examined for precipitins, but they appeared to be completely absent. While the patients with trachoma were studied at the time from another point of view, it is obvious from the frequency of skin reactivity and absence of precipitin that they resemble normal adults in so far as concerns the points dealt with in this study.

Seven laboratory workers engaged in the cultivation of both types of staphylococci, extraction of specific soluble substance, and the

TABLE II

Summary of Skin Tests and Serum Reactions in Adults to Specific Carbohydrates of Staphylococcus

Individuals	Skin tests					Serum reactions		
	No. tested	No. reacting to carbohydrate		Per cent reacting to carbohydrate		No. tested	No. reacting with carbohydrate	
		Type A	Type B	Type A	Type B		Type A	Type B
Normal.....	24	16	0 2 questionable	66	—	5	0	0
Trachoma patients.....	47	30	0 2 questionable	63	—	16	0	0
Laboratory workers.....	7	5	0	71	—	4	0	0
Chronic conjunctivitides...	5	5	0	100	—	4	0	0
Staphylococcus infection...	14	14	0 2 questionable	100	—	6	2	0

handling of these organisms in one way or another, were also subjected to skin tests. Five, or 71 per cent, gave reactions to Type A carbohydrate only, and four of them were tested for circulating precipitins without establishing their presence. Like the trachomatous patients, they resembled normal controls.

Five patients with chronic conjunctivitides of undetermined origin were also given skin tests. All five reacted to Type A, but not to Type B carbohydrate. Upon testing the sera of four of these patients, it was found that all lacked precipitins.

Of 14 patients with verified *Staphylococcus* infection, all manifested

reactions to Type A carbohydrate, while two reacted questionably to Type B. In six of these patients a study was made of the presence of antibodies, and it was found that in two, precipitation occurred with Type A carbohydrate but not with Type B carbohydrate.

Serum Precipitation Reactions

As already indicated, a study was made concurrently of the presence of precipitin in individuals evincing skin reactions to the polysaccharides of *Staphylococcus*. While it was not feasible to test the

TABLE III

Precipitation of Staphylococcus Carbohydrate, Type A, by Serum of Patients with Homologous Infection

Patient and diagnosis	Dilution of Type A carbohydrate						Type B carbohydrate
	*20	50	100	200	500	1,000	
A. Children							
P—osteomyelitis.....	—	+	++	+++±	±	—	—
McF—osteomyelitis.....	+	+++±	++++	+++++	+	—	—
Pr—osteomyelitis.....	+	++	+++	+++	+++	+	—
M—osteomyelitis with amyloid disease.....	++	+++	++++	+++++	++	+	—
A—septicemia.....	—	+	++	+++	+	—	—
B. Adults							
L—septicemia.....	+	++	++++	++	±	—	—
W—osteomyelitis.....	—	++	+++	+++	++	±	—

Precipitation of Type B carbohydrate was tested in same dilutions as for Type A.

* These figures represent dilutions in thousands.

serum of every individual observed, nevertheless a sufficient number of tests were conducted to deserve certain comments. Thus, it was found that none of the individuals who were free of *Staphylococcus* infection possessed precipitins for either carbohydrate despite the presence of definite and, in some instances, marked skin reactivity to one of them. Even during active infection when skin reactivity is also present, antibodies are not necessarily demonstrable in the patient's serum. Thus, of 24 individuals suffering from *Staphylococcus* infection, the sera of only seven contained specific precipitins. The

serum precipitation reactions of these patients are summarized in Table III. Examination of the protocol shows that five of the patients were children, three suffering with osteomyelitis, one with osteomyelitis complicated by amyloid disease, and the fifth with septicemia. The antibody titre for Type A ranged from 1:200,000 to 1:1,000,000. Of these patients, three recovered and two died. One, M, died of the effects of amyloid disease; the other, A, while apparently recovering from the Staphylococcus infection, died suddenly of a terminal Streptococcus complication with septicemia. Of the two adults, one was affected with septicemia, the other with osteomyelitis. The titre in one was 1:200,000, in the other 1:500,000. Both patients recovered. It may be that the occurrence of antibody in the serum is not only an actual measure of the degree of immunity developed by the patient, but that it may comprise a prognostic sign in so far as the Staphylococcus infection is concerned.

Thus, the indications from Table III are that precipitins can be detected in the blood only when the infection is severe, prolonged, or generalized. They have not been found in patients with localized infections (furuncles, infected elbow, infected foot, etc.) even when the infected tissues yielded Staphylococcus predominantly or pure cultures of the organism on cultivation. If an analogy is permissible from experimental infections (4) and artificial immunization (1, 3) with Staphylococcus in rabbits and monkeys, then it can be stated that the serum reactions in the human being might have been anticipated. In this connection, it has already been shown that prolonged intravenous immunization with the heat-killed organisms stimulates precipitins in only a small proportion of animals, and localized infections induced by subcutaneous agar foci containing virulent Staphylococcus, even when repeated two or three times, failed to incite the formation of carbohydrate antibody. So, also, repeated intracutaneous injections of dead staphylococci did not elicit a precipitin response.

Occurrence of Specific Precipitins in the Urine of Patients with Staphylococcus Infection

In several of the patients suffering from the more severe Staphylococcus infections, a study was made of the occurrence of precipitinogen in the urine. Tests were made at different intervals during the course

of infection, but at no time were specific carbohydrates demonstrable by precipitation in rabbit immune serum. It is quite possible that an intracellular situation of the carbohydrate and its presence in minute quantities in the bacterial cell preclude its demonstration in urine. In the few examples in which precipitation reactions of the urine have been recorded (*Pneumococcus* (9), Friedländer's (10, 11), and typhoid bacillus (12)), the polysaccharide was not only elaborated in comparatively large quantities by the organism under study, but it was distributed in the ectoplasm of the bacterial cell, thus permitting more readily elimination of greater amounts of serologically reactive carbohydrates through the kidney.

DISCUSSION

The elaboration of type specific and immunologically active carbohydrates by different strains of *Staphylococcus* has made it possible to study the development of skin sensitivity during the course of infection, with a degree of specificity hitherto unafforded. Skin reactivity to *Staphylococcus* carbohydrates, apparently absent or rare in infants, occurs with increasing frequency to adult life. When one considers the ubiquity of the organism and the prevalence of notably mild and transient infections due to *Staphylococcus*, it is small wonder that the skin undergoes an alteration. The surprising element in the reaction is that practically all the skin sensitivity is towards the Type A carbohydrate. Previous studies have indicated that Type A strains are the pathogenic variety, whereas Type B organisms have not as yet been encountered in *Staphylococcus* infections of man or animal, and it may be that this will explain in part the development of skin sensitivity to Type A polysaccharide. An interesting observation in this connection is that *Staphylococcus* can be cultivated from the conjunctival sac of most patients with trachoma; and while the organisms are usually Type B, the skin sensitivity encountered in these patients is to Type A. From this it is clear that the mere presence of an organism over prolonged periods of time does not establish skin sensitivity. The high frequency of skin reactions to Type A carbohydrate in normal adults, therefore, suggests actual infection with this organism at some preceding time.

While the number of patients with *Staphylococcus* infection studied

was relatively small, skin reactivity to Type A polysaccharide apparently occurred in all instances. Reactions to Type B were rare and they appeared to be nonspecific. The occurrence of skin sensitivity, however, does not necessarily imply presence of circulating precipitins. In fact, antibodies have never been demonstrated in normal individuals, and even during infection less than 30 per cent of the patients were found to possess demonstrable serum precipitins. Moreover, it has not been possible to detect any correlation between intensity of skin reaction and presence of antibody. The data indicate that while skin reactivity may evolve in all varieties of Staphylococcus infection, precipitins are demonstrable in the blood only during severe, prolonged, generalized infection. The complete absence of antibody for Type B polysaccharide supports a previously reported observation, that strains of this type are nonpathogenic under the more commonly encountered conditions (1).

The occurrence of skin reactions to Type A carbohydrate without circulating antibody in certain instances of nephrosis, and their absence in nephritis cannot be explained at the present time. Whether this can be regarded as evidence that nephrosis is a manifestation of Staphylococcus infection, as supposed by certain investigators (13), cannot be stated from the data at hand. This matter constitutes a problem in itself, and it does not affect the results described in the present report.

SUMMARY AND CONCLUSIONS

1. Skin reactions to the type specific carbohydrates of Staphylococcus indicate that cutaneous sensitivity to Type B is either extremely rare or nonexistent.
2. Skin reactions to Type A carbohydrate occur in about 12 per cent of normal infants and children and in 65 to 70 per cent of normal adults.
3. Skin reactions to Type A carbohydrate occur regularly in patients with Staphylococcus infection.
4. Skin reactivity is not associated with demonstrable serum precipitins, for (a) normal individuals, though skin reactive, have not been found to possess them, and (b) not all patients with verified infection develop them.

5. The patients elaborating type specific precipitins, who numbered less than 30 per cent in this study, are those with severe, prolonged, or generalized Staphylococcus infection.

6. Specific carbohydrates have not been found in the urine of patients during Staphylococcus infection.

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QUANTITATIVE STUDIES ON ANTIBODY PURIFICATION

I. THE DISSOCIATION OF PRECIPITATES FORMED BY PNEUMOCOCCUS SPECIFIC POLYSACCHARIDES AND HOMOLOGOUS ANTIBODIES*

By MICHAEL HEIDELBERGER, PH.D., AND FORREST E. KENDALL, PH.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

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Although the partial dissociation of immune precipitates was first reported many years ago and has since been accomplished in a number of instances (1) the methods used have been empirical and no rigid test could be made of the yield or purity of the recovered antibody until the recent development of absolute, quantitative methods for the determination of precipitins (2-4) and agglutinins (5). Felton has since stated that Types I and II pneumococcus specific precipitates, dissolved with calcium or strontium hydroxide and treated with phosphate to precipitate the polysaccharide, yield antibody which is precipitable by the homologous specific polysaccharide to the extent of 80 to 85 per cent (6).

In a paper from this laboratory it was shown that increasing concentrations of sodium chloride resulted in a progressive decrease in the amount of antibody nitrogen precipitated by the specific polysaccharide of Type III pneumococcus (S III) from homologous antisera (7), and that this decrease was reflected by a diminution in both constants of the equation (8) describing the behavior of the serum:

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2}{A} S^2$$

It was also shown that the decrease was not due to increased solubility of the precipitate but to a shift in the reaction equilibrium by which a given amount of S III combines with less antibody in the presence of

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

a high concentration of salt than under ordinary conditions. Since the precipitin reaction between S III and antibody has been found to be reversible (9) it seemed that the salt effect might also be reversible. In this event a theoretical basis would be available for the hitherto empirical method of dissociating specific precipitates by means of strong salt solutions. On this basis it should be possible, starting with a washed S-antibody precipitate obtained at a proper point in the reaction range, to dissociate a portion of the antibody with strong salt solution and use the residual precipitate repeatedly for the absorption of additional quantities of serum and dissociation of the antibody taken up.

It will be shown below that these expectations were realized in some measure. It was found possible, with Types I and II antipneumococcus horse sera and Type III antipneumococcus rabbit sera to pass from unconcentrated sera in a single step to antibody solutions of which 85 to 93 per cent of the total nitrogen was immune nitrogen. From the extracted precipitate by a modification of the Felton dissociation procedure (6) additional antibody of a similar degree of purity could be obtained.

Experimental

Unless otherwise stated all sera used were absorbed with C substance (10) and with pneumococcus protein (*cf.* 5) in order to remove most of the antibodies other than anticarbohydrate.

1. *Preliminary Experiments on Variation of Hydrogen Ion and Salt Concentration of Extracting Solution.*—Experiments on the effect upon the specific precipitate of buffer solutions of varying pH and of sodium chloride solutions of various strengths are summarized in Table I. 1.5 to 3.0 ml. portions of the rather dilute Felton (11) Type III pneumococcus antibody solution (horse) and 1.0 ml. portions of rabbit 3.50₁ Type III antipneumococcus serum (1:2) and rabbit 3.88₂ anti-egg albumin (Ea) serum (1:1) were used. In the case of Pneumococcus III antibodies, S III prepared according to Reference 12 was added in amounts just sufficient to reach the beginning of the equivalence zone (8), while in the anti-egg albumin serum, Ea was added in amounts sufficient to reach the same point. In the experiments designated series A, however, 1.0 ml. portions were precipitated with a smaller amount of Ea containing 0.049 mg. N. After 1 hour in the cold the tubes were whirled in a refrigerating centrifuge,¹ and the supernatants (series B) were precipitated at room temperature with one-half the amount of Ea. Both series of

¹ Manufactured by the International Equipment Co., Boston.

tubes were washed once with 5 ml. of chilled saline, and were used after centrifugation in the cold. In these and in the S III series 5 ml. of the appropriate buffer or salt solution were added. After incubation at the temperature indicated, with frequent stirring, the tubes were centrifuged and the nitrogen in the supernatants was determined by the micro Kjeldahl method, deducting the small amount in a control sample extracted with 0.9 per cent sodium chloride solution.

The pH measurements were made with the glass electrode.² The pH 3.95 solution was a phthalate buffer prepared according to Clark,³ while the pH, 8.96, 9.84, and 11 buffers were borate solutions, prepared according to Clark.⁴ The concentration of the pH 3.73 buffer, which contained 24.5 gm. crystalline sodium acetate, 48.5 gm. glacial acetic acid and 48.0 gm. sodium chloride per liter, was molar in respect to both sodium and acetate.

Equilibrium was probably reached rapidly in the extractions, as a second treatment with the same solvent rarely yielded much more nitrogen than did the control tube with 0.9 per cent saline. Precipitates from which considerable amounts of nitrogen had been extracted were, however, more gelatinous than at the start, and this possibly contributed to the smaller effect of a second extraction. In many instances it was necessary to filter the supernatant through a small Munktell No. 1F paper in order to remove suspended particles.

2. *Dissociation of Specific Precipitates with Strong Sodium Chloride Solution.*—10 to 50 ml. of type specific antipneumococcus serum or Felton antibody solution (11) were chilled, diluted with 2 to 3 volumes of chilled 0.9 per cent saline, and precipitated with an amount of homologous specific polysaccharide (12) calculated to bring the system to the beginning of the equivalence zone (8) or to leave a small amount of antibody. After the precipitate had flocked, the mixture was centrifuged in the cold and the precipitate evenly suspended and washed with 10 to 50 ml. portions of chilled saline until the amount of heat coagulable protein extracted was at a minimum. The residue was then evenly suspended in a volume of 10, 15, or 20 per cent (gm. per 100 ml. solution) sodium chloride solution equal to that of the original serum and immersed in a water bath at about 30°, with frequent stirring. After 1 hour the mixture was centrifuged and the supernatant filtered through a small Munktell 1F filter paper if necessary to remove suspended particles. The solution, which was generally water clear, was dialyzed in the cold in cellophane tubing against repeated changes of 0.9 per cent salt solution until these gave the same interferometer⁵ reading as did a control sample of the salt solution used. In general the dialyzed solutions from the 10 per cent salt extractions remained clear, while small amounts of precipitate settled from the 15 per cent

² By Mr. F. Rosebury of the Department of Biological Chemistry.

³ Clark (13), page 200, pH 4.

⁴ Clark (13), pages 208 and 209.

⁵ Purchased through a grant from the Bache Fund of the National Academy of Sciences.

salt extracts and larger precipitates from the 20 per cent salt extracts, owing, doubtless, to the actual solvent action of salt solutions of this strength superimposed on their effect of shifting the S-antibody equilibrium. The 15 per cent salt solution was used in most instances. The rabbit antipneumococcus extracts were usually dialyzed under negative pressure in order to reduce the final volume. This was desirable in connection with the analytical control of the solutions, owing to the appreciable solubility of rabbit S-anti-S precipitates. At the end of the dialysis the solutions were centrifuged if necessary, preserved by the addition of 1 per cent by volume of 1 per cent merthiolate solution, and used for analysis after 24 hours, after centrifugation to remove any additional precipitate caused by the merthiolate.

Analyses for precipitin nitrogen were made according to References 2, 3, and 4 by addition of a slight excess of homologous S to duplicate 2 to 5 ml. portions of the chilled solution and determination of the amount of nitrogen in the washed precipitate after 48 hours in the cold. Since the total nitrogen in the supernatants was extremely low, one washing with 3 ml. of chilled saline was considered sufficient. Blank tubes were run under the same conditions with the same amount of antibody solution and micro Kjeldahl determinations were run separately on the supernatants (plus washings) of the blank tubes and the generally negligible residues,⁶ the sum of the two giving the total nitrogen of the antibody solution. In the case of S I the small amount of S I nitrogen precipitated was deducted from the total nitrogen precipitated in order to give antibody nitrogen. As large aliquot portions as possible of the supernatants from the precipitin determinations were analyzed for agglutinin nitrogen according to Reference 5, by addition to a measured volume of a suspension of homologous type specific pneumococci, and determination of the increase in nitrogen over that in the pneumococcus suspension alone after centrifugation and washing once. It was also possible to analyze the supernatants from the centrifuged pneumococci for nitrogen and determine agglutinin N by difference. Both methods of analysis gave the same result in the case in which a comparison was made. Control determinations on Type I and Type III pneumococci with as much as 1 ml. of undiluted normal horse serum showed no absorption of nonspecific nitrogen, while the small amount taken up by the Type II suspension was deducted from the agglutinin nitrogen actually determined in the Type II antibody supernatant.

The analytical data are summarized in Table II. Values are also given in the table for the amount of antibody nitrogen taken, the amount and percentage recovered, and the percentage of the total nitrogen in the recovered solutions accounted for as antibody nitrogen. In the case of antisera the precipitin content was taken as "antibody," since it had been shown for Type I antipneumococcus

⁶ These residues, when present, appeared to be due to the film of antibody protein remaining in the pipette and generally appeared only after pipettes were refilled. The small amount of nitrogen thus precipitated was deducted from the precipitable nitrogen found.

sera that the anticarbohydrate precipitin and agglutinin content are identical (14). In both Felton antibody solutions (*cf.* also 14) and in those now reported, a portion of the antibody could only be recovered as agglutinin.

In the first series of experiments summarized in Table II, 64 ml. of a Type I antipneumococcus Felton solution, B 79, containing 2.22 mg. of total N and 1.09 mg. of N precipitable by S I, per ml., were diluted and precipitated at 0° with 8 mg. of S I. The washed precipitate was first treated with 15 per cent sodium chloride solution and the recovered antibody designated 79 A. After analysis the solution was run through a Chamberland L₂ filter and again analyzed (79 A₁). The specific precipitate remaining after the salt extraction was washed in the cold with 25 ml. of water, centrifuged in the cold, and then evenly suspended at 0° in 30 ml. of unabsorbed New York State Type I antipneumococcus horse serum 444⁷ containing 1.92 mg. of anti-S I N per ml. After letting stand in the refrigerator overnight, the mixture was centrifuged in the cold and the first 50 ml. of 0.9 per cent saline washings were added to the supernatant serum. After an additional washing the precipitate was extracted for 1 hour at 30° with 30 ml. of 15 per cent saline. The extract (79 B) was dialyzed as described above and the precipitate washed with water and treated again in the same way with the partially exhausted serum to yield antibody solutions 79 C and D. The remaining precipitate was then dissociated further by means of the method described in the following section. The various solutions obtained are numbered 1 to 5 in Table II to indicate successive treatments of the same precipitate.

The Type II specific precipitate used was derived from the pooled serum of horses which had been immunized simultaneously to Types I and II pneumococci by addition of somewhat less S II to the unabsorbed serum than was calculated as necessary to remove all of the antibody. After thorough washing equal portions of this precipitate were dissociated with 15 per cent (portion A) and 20 per cent (portion B) salt solution. Data on the recovered antibody solutions are given in Table II. The surprising observation was made that these solutions contained much anti-S I as well as anti-S II, a point which will be taken up in the discussion.

In the case of Type III antipneumococcus specific precipitates from antisera obtained from the horse, the recovered antibody solutions were of a considerably lower degree of purity than in the case of Types I and II, and also contained relatively less precipitin and more agglutinin. Efforts will be made to improve on these results. Preparation 792 B was derived from absorbed whole serum, while 792 C was prepared from a Felton solution made from the same serum. Type III antipneumococcus rabbit sera, however, readily yielded antibody solutions of which over 90 per cent of the nitrogen was immune nitrogen. It will be noted throughout that regardless of the type of antiserum, there is no advantage in starting with partially purified antibody, such as Felton solutions.

3. Dissociation of Type I and Type III S-Anti-S Precipitates by Means of Barium

⁷ Kindly supplied by Dr. Augustus B. Wadsworth.

Hydroxide and Barium Chloride.—This modification of Felton's dissociation procedure (6) was suggested by the insolubility of the barium salts of S I (15) and S III (12). The Type I antipneumococcus precipitate which had been used four times for dissociation was washed with cold water and suspended in 25 ml. of water at 0°. 0.1 normal barium hydroxide was then added drop by drop until no more of the precipitate appeared to dissolve, about 1 ml. being necessary. To the strongly alkaline solution were added 4 ml. of 10 per cent barium chloride solution containing 1 per cent by volume of the same barium hydroxide solution. This increased the amount of precipitate, and as it settled readily, leaving a clear solution, the mixture was centrifuged in the cold after 5 minutes. After an additional 10 minutes the clear supernatant was made very faintly acid to litmus with dilute acetic acid and dialyzed against 0.9 per cent sodium chloride solution until only traces of barium ion remained. These were removed after addition of 1 per cent merthiolate solution by means of 0.6 ml. of 2 per cent sodium sulfate solution. After 24 hours in the cold the centrifuged solution was subjected to analysis and proved to be highly pure antibody (Table II, 79 E).

Type III S-anti-S precipitates from horse serum were worked up similarly, but did not yield antibody of the same degree of purity. Preparation 792 D, obtained from an antibody solution, contained a somewhat higher proportion of immune nitrogen than did a corresponding preparation, E, from absorbed whole serum.

DISCUSSION

In continuation of our work on a quantitative theory of the precipitin reaction, the observation was made (7) that a given amount of pneumococcus specific polysaccharide combined with less antibody in the presence of high sodium chloride concentrations than under normal physiological conditions. Further study showed that this shift in the reaction equilibrium was reversible, and that a theoretical basis was thus provided for the dissociation of certain specific precipitates by means of strong salt solutions. From the data given it is apparent that a rapid, simple, and in several instances, readily reproducible method is available for the preparation, in a single step from unconcentrated antiserum, of pneumococcus anticarbohydrate in a high state of purity.

In the preliminary experiments summarized in Table I it was found that pneumococcus S-anti-S precipitates formed at 0.9 per cent salt concentration gave up far more nitrogen to strong salt solutions than did egg albumin-antibody precipitates, in agreement with observations on the reverse reaction (7) showing that the amount of nitrogen pre-

precipitated in the egg albumin-antibody reaction was almost the same in strong or weak sodium chloride solutions, while in the S-anti-S system far less antibody was precipitated in the presence of strong salt. On the other hand, the egg albumin precipitates proved to be more sensitive than S-anti-S to the action of acid (pH 4) or relatively

TABLE I

Effect of Hydrogen Ion and Salt Concentration on Amount of Antibody Nitrogen Extracted from Specific Precipitates

Antibody N or total N* in washed ppt.	Temperature of extraction	Phthalate, pH 3.95	HOAc-acetate NaCl buffer μ Na μ OAc pH 3.73	Borate, pH 8.96	Borate, pH 9.84	Borate, pH 11†	μ (5.85 per cent) NaCl solution	10 per cent NaCl solution	15 per cent NaCl solution	20 per cent NaCl solution
mg.	degrees	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Pneumococcus Type III Antibody Solution, B 66, Horse										
0.55	0, 3 hrs.	0.00		0.01	0.03	0.06				
1	28.5-30							0.35	0.43	0.47
Pneumococcus Type III Antiserum, 3.50 ₁ , 1:2, Rabbit										
1.2	21		0.26				0.11	0.19		
1.1	28-30							0.40	0.44	0.43
Anti-Egg Albumin Serum, 3.88 ₂ , 1:1, Rabbit										
1.8	0, 2 hrs.	0.35†		0.03	0.07	0.34				
0.62 (series A)	21-22	0.13§			0.08	0.35				
0.16 (series B)	21-22	0.08			0.06	0.16				
0.88	22-23		0.46				0.03	0.04		
0.81	28-30							0.06	0.05	0.04

* In Ea-anti-Ea system only.

† Calculated from quantities of components used.

‡ Second and third extractions removed 0.13 and 0.03 mg. N.

§ Extraction at pH 10 after removal of N extractable at pH 4 failed to increase the amount extracted, nor did changing to pH 4 after 2 extractions at pH 11.

|| A second extraction removed 0.04 mg. N.

strong alkaline (pH 11) buffers. In the pH 4 buffer, at least, the effect was mainly one of solvent action, rather than a shift in the proportions of the components, since neutralization of the solution resulted in precipitation. When the anti-egg albumin was fractionally precipitated and the two fractions were tested separately with the buffers, the second fraction (Table I, series B) was found far more

soluble than the first (series A), an additional confirmation of the presence of more than one antibody in antisera to this crystalline, homogeneous antigen (16).

The data on the actual isolation of purified antibody by dissociation of washed pneumococcus S-anti-S precipitates are given in Table II.

TABLE II
Analyses of Antibody Solutions Obtained by Dissociation of Specific Precipitates

Analyses of Antibody Solutions Obtained by Dissociation of Specific Precipitates									
Serum or antibody	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Percentage recovery of antibody N	Total N per ml. of recovered antibody solution		Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N
				mg.	mg.				per cent
Type I Antipneumococcus, Horse									
1. 79A	70 (P)*	21.6 (P)	31 (P)	0.323	0.261	0.029	0.290		90
A1				0.300	0.236	0.024	0.260		87
2. B	57.3	7.5	13.1	0.177	0.138	0.018	0.156		88
3. C		4.7	8.2	0.116	0.089	0.009	0.098		85
4. D		3.0†	5.2	0.076	0.061	0.003	0.064		84
5. E		9.3 (P)	13.3 (P)	0.227	0.217	0.005	0.222		98
Type II Antipneumococcus, Horse (Pn I, II)									
NYC I, IIA	108	21.1 (P)	19.5 (P)	0.367	0.321†				87 (P)
NYC I, IIB	108	24.2	22.5	0.358	0.321†	0.008	0.329		92
Type III Antipneumococcus, Horse									
792 B	32.5	3.1	9.5	0.142	0.074	0.025	0.099		70
792 C	43.8	3.5	8	0.092	0.048	0.022	0.070		76
792 D	14.4	2.8	19.5	0.159	0.127	0.014	0.141		89
792 E	37	4.4	11.9	0.159	0.110	0.016	0.126		79
Type III Antipneumococcus, Rabbit									
3.50 ₁	18.0	4.6 (P)	25.5	0.113	0.102				90 (P)
3.51 ₁	72	16.5	23	0.433	0.383	0.020	0.403		93

by (P) refer to precipitin N only.

serum 444 up to this point. Analysis

* Values followed by (P) refer to precipitin N only.

† 15.2 mg. of antibody N recovered from serum 444 up to this point. Analysis of the serum supernatant showed that 23.7 mg. had been removed.

‡ Separate determinations with S II, followed by S I, gave anti-S II, 0.237, 0.239, and anti-S I, 0.081 and 0.076 per ml. for the two solutions.

It is seen that the dissociation by strong salt solutions of precipitates obtained directly from whole sera, or sera absorbed with C substance and pneumococcus protein, yields antibody solutions of as high a degree of purity as does dissociation of precipitates originally derived from partially purified antibody solutions.

The five Type I pneumococcus antibody solutions given in the table were obtained by successive dissociations of a single S-anti-S precipitate. Between all dissociations except the last two this precipitate was used again for the removal of additional quantities of antibody from an unabsorbed whole serum. After the fourth dissociation, which yielded relatively little antibody, an additional amount, of exceptionally high purity, was recovered by the barium method described in the experimental part. If this portion is considered as derived from the original precipitate 44 per cent of the precipitin in the Felton antibody solution used for its formation was recovered, as well as 26 per cent of the antibody in the serum used for the second, third, and fourth absorptions. After these absorptions 59 per cent of the precipitin content was still present in the serum. Attempts will be made to improve on the yields of recovered antibody.

Although one of the recovered Type I solutions may be said to be 95 to 100 per cent pure antibody within the limits of error of the analytical methods used, it is apparent that even the simple procedure used in this and the other cases has resulted in a slight change in the properties of the antibody. Thus it has been shown that anticarbohydrate in Type I antipneumococcus sera is entirely precipitable by the homologous specific polysaccharide (14), while the anticarbohydrate in Felton solutions (14, 17), as well as in the more highly purified solutions now reported, is in part recoverable only as agglutinin.

In the experiments on the recovery of antibody from an S II-anti-S precipitate obtained from a combined Type I, II antipneumococcus horse serum, the surprising discovery was made that the recovered solution, although prepared from a precipitate formed by addition of S II (12) to the serum, contained significant amounts of antibody to the specific polysaccharide of Type I pneumococcus. Since Types I and II anticarbohydrate do not cross react with the heterologous polysaccharides when tested separately, it is believed that the cross reaction in the combined serum is another instance (*cf.*, for example, 16) in which the antibody in question contains too few reactive groupings in its molecule to take part by itself in the building up of aggregates large enough to precipitate, but may add to an aggregate in process of formation between antigen (or hapten) and antibody which are multivalent with respect to each other. The alternative explana-

tion, that the anti-S I and anti-S II groupings are on the same molecule is excluded by the experiment recorded in the footnote to Table II, in which it is shown that the anti-S I is left in the supernatant when the purified antibody is precipitated with S II. That this is possible in the recovered antibody solution although the anti-S I was firmly bound in the original precipitate is probably due to an equilibrium between free and bound anti-S I.

While the results with Type III antipneumococcus horse serum are the poorest of the series as to yield and quality of the recovered antibody, it is believed that they are better in the latter respect than those obtained by any other single step procedure now available. Attempts will be made to improve on the S III-anti-S results. The dissociation method proved applicable, however, to the corresponding rabbit antisera, and it is probable that both of the solutions described in the table represent 95 to 100 per cent pure antibody, since rabbit S-anti-S precipitates are more soluble than those formed with antibodies produced in the horse and no solubility correction has been applied to the figures in the table.

Felton (6) has reported the preparation, from Types I and II pneumococcus antibody solutions, of zinc and aluminium containing solutions of which 100 per cent of the protein was precipitable by the homologous specific polysaccharide. After removal of the metal salts, the remaining protein was still precipitable to the extent of 80 to 90 per cent. Felton has also reported 80 to 85 per cent precipitable antibody by an alkaline earth, hydroxide-phosphate dissociation method. Chow and Goebel (18), also starting with partially purified Type I pneumococcus antibody, have stated that their end-products were, under optimal conditions, precipitable to the extent of 85 to 90 per cent.

Thus, although antibody of the degree of purity attained by the present method has already been reported, the methods used are not simple of execution and cannot be carried out on the original serum, two disadvantages which are eliminated by the salt dissociation method herein described. Moreover, analytical data are now presented for the first time on antibody of over 80 per cent purity.

A study of the physical, chemical, and immunological properties of the new antibody material is under way.

The authors wish again to express their thanks to Dr. Torsten Teorell, with whose assistance many of the observations (7) were made which led to the present work.

SUMMARY

1. Quantitative data are given on the effect of changes in hydrogen ion concentration and of salt solutions of high concentration on certain immune precipitates obtained at lower salt concentration.

2. Advantage is taken of the shift in reaction equilibrium brought about by the salt in the case of pneumococcus carbohydrate-anti-carbohydrate precipitates to enable the preparation, in a single step from unconcentrated serum, of antibody solutions in which up to 93 per cent of the total nitrogen is immune nitrogen. The method permits successive absorptions of a serum to be made with the same specific precipitate.

3. A modification of Felton's alkaline earth hydroxide dissociation procedure is proposed which yields highly purified antibody with precipitates which have been subjected to several successive salt dissociations.

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SURVIVAL OF THE VIRUS OF POLIOMYELITIS IN THE ORAL AND NASAL SECRETION OF CONVALESCENTS*

By S. D. KRAMER, M.D., A. E. SOBEL, PH.D., L. H. GROSSMAN,
AND B. HOSKWITH

*(From the Laboratories of the Infantile Paralysis Commission of the Long Island
College of Medicine, and the Jewish Hospital, Brooklyn, New York)*

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Certain epidemiologic concepts of poliomyelitis still rest on very sparse data. The detection of the virus in the oral and nasal secretions and tissues of living human beings is necessary to substantiate some of these concepts. In that category belong the detection of a carrier in the normal healthy population to account for the widespread immunity to poliomyelitis (1) and the detection of the virus in the nasal secretions of the so called mild characteristic illness of Paul and Trask (2), occurring in close proximity to frank cases of the disease. The data presented below attempt to answer a similar, though perhaps a somewhat more practical epidemiologic question, namely, how long does the virus of poliomyelitis persist in the nasal and oral secretions after the onset of the disease?

The number of times the virus has been isolated from the oral and nasal cavities of human beings, is very small. In a recent publication Paul, Trask and Webster (3) review the literature and estimate that the virus has been isolated 13 times from the nasal and oral secretions and tonsils of living human beings. Of these, 8 positive results were obtained from patients during or prior to the acute stage of the disease; only 2 from convalescents, one 17 days (4) and a second 4 months (5) after the onset of illness; 2 (6, 7) from healthy contacts, and one (1) from the tonsils and adenoids of a child without any history of contact with a case.

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In this paper we wish to record 2 additional instances of successful isolation of the virus of poliomyelitis from 2 recovered patients 16 and 13 days respectively after the onset of the illness, and to describe a simple method for the bacterial sterilization and the concentration of nasal washings.

A number of methods have been employed for obtaining and treating oral and nasal secretion preparatory to inoculation into animals. A major problem in the preparation of the inoculum has been the sterilization of the heavily infected material. This has been accomplished by the different authors in a variety of ways; by filtration through a Berkefeld candle; by treatment with ether; by phenolization or by glycerolation.

Since the amount of virus in the secretion is probably small, attempts have been made to concentrate the material either by vacuum distillation at 35–38°C. or by ultrafiltration. In most instances the concentration has reduced the volume from a quarter to one-tenth of the original quantity. The volume and type of fluid employed has varied. Kling and Pettersson (7) employed large quantities of sterile water (1 to 2 liters), reducing this large volume by vacuum distillation to from 100 to 200 cc. Taylor and Amoss (8), Paul and Trask (9) and Flexner, Clarke and Fraser (10) used quantities varying from 30 to 150 cc. Amoss and Taylor (11) carried their concentration to a final volume of 2 cc. Positive takes have been reported by the use of all these methods, though the takes have been few in number. When filtration was not employed, brain abscesses have resulted from time to time.

A somewhat modified, and we believe simplified, technique described below yielded sterile material concentrated to sufficiently small volume to permit intracerebral inoculation. Efforts were made to obtain a maximum amount of virus by the use of moderately large quantities of sterile water and the procedures followed for sterilization and concentration were those considered least likely to be injurious to the virus.

EXPERIMENTAL

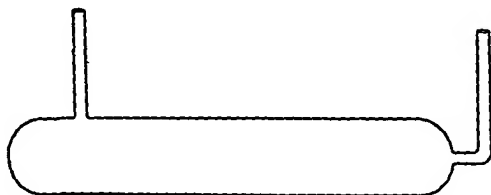
Nasal washings were performed on patients¹ during the acute stages of the illness and at varying intervals after the acute symptoms had subsided. 50 to 75

¹ The patients were obtained from the poliomyelitis wards of the Kingston Avenue Hospital in Brooklyn, New York.

cc. of sterile distilled water was introduced by means of a soft rubber catheter passed into one nostril, and the nostril gently compressed against the catheter to prevent back flow and leakage. The water was slowly forced through the catheter with a 50 cc. syringe. With the patient lying prone, and the head held somewhat to one side, little difficulty was encountered in obtaining the return of most of the water introduced. The flow from the opposite nostril and the mouth of the patient was caught in a sterile pus basin.

The amount of the washing was measured, 10 per cent of anesthetic ether added, well shaken for 5 to 10 minutes and left overnight in the ice chest. The following day the washing was placed in a specially prepared sterile glass container, as shown in Text-fig. 1, and attached by glass seal to a vacuum system. The containers were then dipped in an acetone-CO₂ mixture until frozen solid, and desiccation accomplished by means of a hi-vac pump in series with a mercury pump.

Throughout the period of desiccation, the washings were kept frozen. While no accurate check was made of the exact temperature of the frozen material, the vacuum reading on a McCleod gauge varied from 0.03 to 0.001 mm. of pressure after all the ether had been volatilized. From 4 to 6 hours were required to re-



TEXT-FIG. 1

duce the volume of the washings to 1 cc. or less. The container was then sealed off under vacuum. The concentrated material was removed by breaking one of the sealed ends and poured off aseptically into a test tube. The mucoid material adhering to the sides of the container was emulsified with a little sterile distilled water by rubbing a sterile pipette gently along the sides of the container. The total amount, usually 2 to 4 cc., was then inoculated intracerebrally into a normal animal, a small portion being retained for culture. The cultures (aerobic) were sterile on blood plates and in broth and there was no instance of brain abscess.

A series of controls were included. These consisted of from 0.05 to 0.2 cc. of 5 per cent suspension of stock virus, diluted to 50 cc. with sterile water and treated with ether and desiccated in the same manner as the washings. Our technique was considered adequate when these controls yielded positive takes in test animals in at least 3 consecutive desiccations (Table I).

20 nasal washings including 2 from physicians in charge of the patients and 3 second or repeat washings of children were studied

TABLE 1

Results of Intracerebral Inoculation of Concentrates in Rhesus Monkeys

Results of Intracerebral Inoculation of <i>Conc.</i>													Outcome			
Case	Patient	Age	Date of onset		Date of nasal washing		Time from onset to washing		Date of inoculation		Amount of inoculum	Monkey No.	Fever*	Symptoms	Lesions	Neutralization test
			1935		1935		days		1935							
1	G. B.	5 yrs.	Sept. 27	Sept. 30	3	3	Oct. 2	2	J2	—	—	—	—	—	—	Failed to neutralize
2	S. D.	6	Oct. 5	Oct. 14	9	9	Oct. 16	3.6	J2-3	—	—	—	—	—	—	
3	A. S.	10	Oct. 3	Oct. 14	11	11	Oct. 16	3.4	J2-5	+22	Generalized weakness. Refused to climb	Not sacrificed	—	—	—	
4	F. G.	9½	Oct. 2	Oct. 15	13	13	Oct. 17	3.9	J2-7	—	Monkey's right arm paralyzed. Animal sacrificed	Typical experimental poliomyelitis	—	—	—	Failed to neutralize
5	H. LaG.	8	Sept. 30	Oct. 15	16	16	Oct. 17	3.5	J2-8	+8	Left upper limb weak. Animal sacrificed	Suggestive but not typical of experimental poliomyelitis	—	—	—	
6	R. McK.	13	Sept. 14	Oct. 22	36	36	Oct. 24	2	J6-6	+28	—	—	—	—	—	
7	J. S.	13	Oct. 16	Oct. 22	6	6	Oct. 24	2	J6-7	—	—	—	—	—	—	Hemorrhage, infiltration, satellitosis, neuronophagia. Disappearance of ganglia
8	M. B.	8	Oct. 8	Oct. 24	16	16	Oct. 26	1.6	J6-9	—	—	—	—	—	—	
9	R. B.	7	Oct. 4	Oct. 24	20	20	Oct. 26	1.6	J7-0	—	—	—	—	—	—	
10	J. H.	13	Sept. 22	Oct. 29	37	37	Oct. 31	1.8	J7-3	—	Weakness of right arm. Animal sacrificed	—	—	—	—	
11	D. A.	5	Oct. 26	Oct. 29	3	3	Oct. 31	1.5	J7-5	+21	—	—	—	—	—	
12	R. C.	14	Oct. 16	Oct. 29	13	13	Oct. 31	3	J7-6	—	—	—	—	—	—	
13	R. F.	4	Oct. 11	Oct. 30	19	19	Nov. 1	1.5	J9-8	—	—	—	—	—	—	

14	G. B.	5	Oct. 8	Oct. 30	22	Nov. 1	2	J9-9	+10	All limbs weak. Monkey sacrificed	Suggestive but not typical of experimental disease	Neutralized stock virus on 2 occasions
15	I. F.	6	Sept. 22	Nov. 4	43	Nov. 6	1.2	J1-03	+5	Monkey appeared sick. Hind limbs weak. Tremors, but no definite paralysis	—	—
16	M. B.	8	Repeat	Nov. 19	22	Nov. 21	1.5 cc. intracer., 2.5 cc. intraper.	J1-22	—	—	—	—
17	G. B.	5	Repeat	Nov. 19	42	Nov. 21	1.5 cc. intracer., 2.5 cc. intraper.	J1-23	—	—	—	—
18	H. LaG.	8	Repeat	Oct. 30	31	Nov. 1	1.4	J9-7	+10	General weakness. Disinclination to climb	Not sacrificed	Failed to neutralize
19	K.**			Nov. 4		Nov. 6	2	J1-02	+9	Refused to walk or climb. General weakness	—	Failed to neutralize
20	R.**			Nov. 4		Nov. 6	2.3	J1-01	+5	Generalized weakness	—	Failed to neutralize

Controls

Monkey No.	Date of inoculation	Amount of inoculum	Outcome
J3	1935		
J2-4	Oct. 2	2 cc. concentrate containing 0.05 cc. 5% suspension of virus	Poliomyelitis, Oct. 9, 1935
J2-6	Oct. 16	3.8 cc. concentrate containing 0.2 cc. 5% suspension of virus	Poliomyelitis, Oct. 24, 1935
J6-5	Oct. 17	1.8 cc. concentrate containing 0.2 cc. 5% suspension of virus	Poliomyelitis, Oct. 25, 1935
	Oct. 24	2 cc. concentrate containing 0.2 cc. 5% suspension of virus	Poliomyelitis, Nov. 15, 1935

*+ Indicates fever. Numeral indicates day of fever.

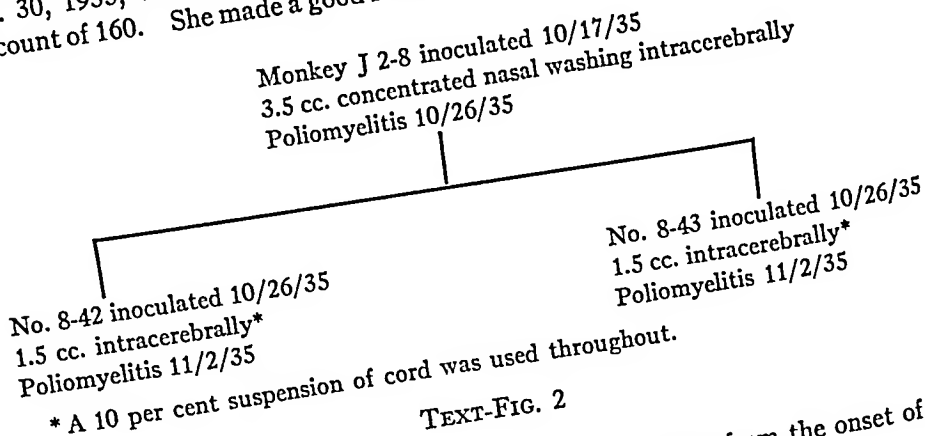
** Adult contacts.

(Table I). 2 positive takes were obtained, and the virus subjected to passage (Text-figs. 2 and 3).

The $3\frac{1}{2}$ cc. of concentrated nasal washing from which the first strain was isolated, was obtained from a child, H. La G., 16 days after the onset of illness.

The concentrate was inoculated intracerebrally into monkey J2-8 on Oct. 17, 1935. On Oct. 26 the right upper extremity was paralyzed. The animal was sacrificed on that day and $1\frac{1}{2}$ cc. of a 10 per cent suspension of the cord was inoculated intracerebrally into 2 normal monkeys (8-42, 8-43). On Nov. 2 both these animals developed rapid progressive paralysis, were prostrated the same day and were sacrificed. The histologic sections of the cords of all 3 animals were typical of experimental poliomyelitis.

The hospital record of the child, H. La G., reveals that she had become ill on Sept. 30, 1935, with typical symptoms and physical findings and a spinal fluid cell count of 160. She made a good recovery without residual paralysis. A second

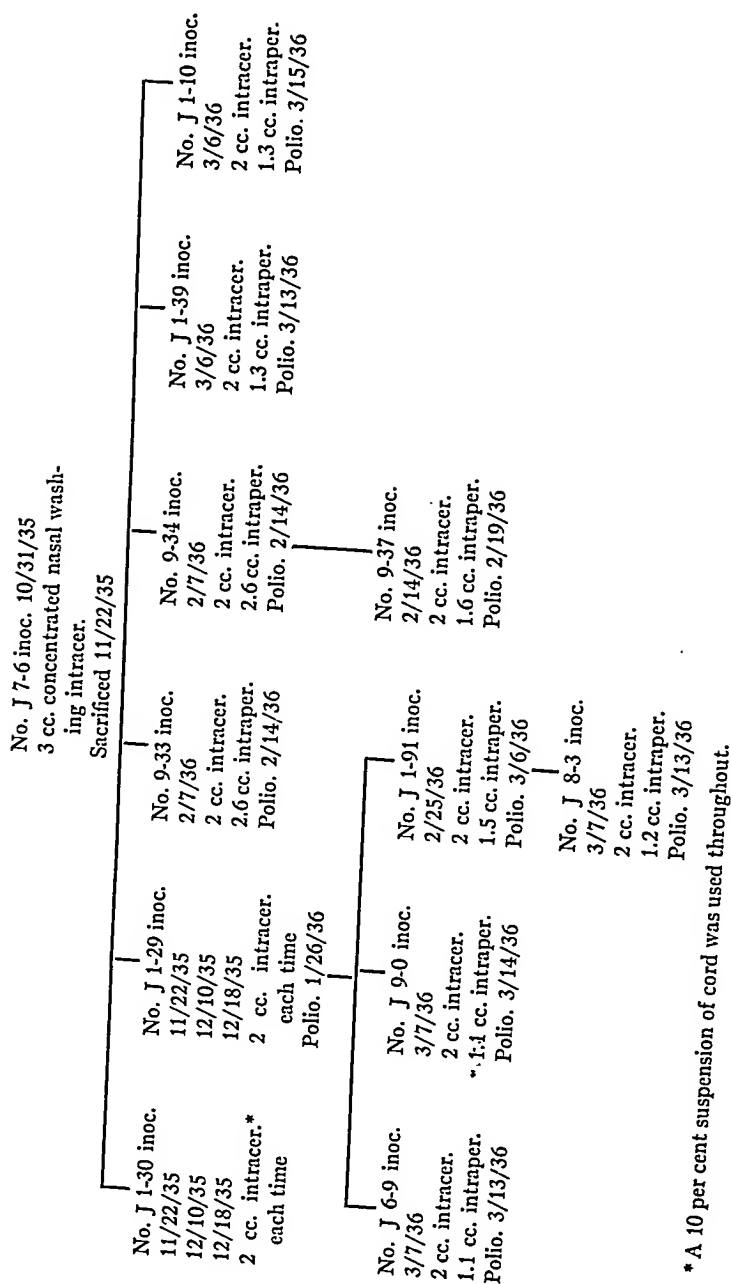


TEXT-FIG. 2

nasal washing of this child taken 15 days later or 31 days from the onset of the illness yielded a negative result.

The second positive take was obtained from the nasal washings of a 14 year old boy, R. C.

This boy became ill Oct. 16, 1935, and was admitted to the hospital already paralyzed. Nasal washings were obtained on Oct. 29, or 13 days after the onset of illness, when the patient was convalescing. 3 cc. of the concentrated washing was inoculated intracerebrally into monkey J7-6 on Oct. 31, 1935. The animal had an elevation of temperature to 105°F . on Nov. 21, appeared ill and weakness of right arm was noted. The following day the temperature returned to 102.4°F . and the animal was sacrificed. Histologic study of the cord showed many hemor-



* A 10 per cent suspension of cord was used throughout.

TEXT-FIG. 3

rhages in the white and grey matter, moderate infiltration with lymphocytes, mononuclear and a few polymorphonuclear leucocytes. Many of the anterior horn ganglion cells showed degenerative changes, some satellitosis and neuronophagia.

Somewhat greater difficulties were encountered in subjecting this strain to further passage.

On the day the animal was sacrificed a 10 per cent suspension of cord was prepared and 2 cc. of the suspension was inoculated intracerebrally into 2 animals, (J1-29 and J1-30). Monkey J1-29 showed fluctuations in temperature between 102° and 105°, but not evidence of any palsy. Both animals were reinoculated with 2 cc. each of a 10 per cent suspension of the cord on Dec. 10, 1935, and again on Dec. 18. Monkey J1-30 remained unaffected by these inoculations, whereas monkey J1-29 continued to show fluctuations in temperature from time to time until Jan. 26, 1936, when the animal developed weakness of all four extremities and was sacrificed. A histologic study of the section of the cord showed extensive degenerative changes in the ganglion cells, and moderate inflammatory reaction. The findings were compatible with a diagnosis of poliomyelitis, but not typical of the experimental disease. Monkey J1-91 inoculated with a suspension of cord of this animal (J1-29) yielded typical poliomyelitis. A further passage from monkey J1-91 resulted in typical poliomyelitis in monkey J8-3.

2 additional animals (9-33 and 9-34) were again inoculated with a 10 per cent suspension of the original cord from monkey J7-6 on Feb. 7, 1936. 2 cc. was inoculated intracerebrally and 2.6 cc. was inoculated intraperitoneally into each of the animals. Both of these animals developed typical poliomyelitis. Injection of 2 cc. intracerebrally and 1.6 cc. intraperitoneally of a 10 per cent suspension of cord from monkey 9-34, yielded typical poliomyelitis in monkey 9-37. Histologic study of the 3 cords showed typical experimental poliomyelitis. Because of the unusual response of monkey J1-29, considerable effort was made in establishing this strain of virus in the monkey and Text-fig. 3 illustrates these efforts.

7 other animals, which received various inocula, presented suggestive temperature rises or generalized weakness (Table I).

These animals were healthy, vigorous animals at the time of the inoculation, had no cough or diarrhea. Within 1 to 3 weeks following inoculation, they developed elevations in temperature and appeared ill. They failed to evidence the normal activity of the healthy monkey, refused to run or jump and tired easily. Within 24 hours of the elevation in temperature 2 of these animals (J6-6, J9-9) were sacrificed and a 10 per cent suspension made from part of the cord and inoculated intracerebrally into 4 animals (2 for each suspected animal). The histologic pictures in both animals were suggestive of, and compatible with a

diagnosis of poliomyelitis, but were not typical of the experimental disease. We have thus far been unsuccessful in obtaining a positive take from either of these cords. The remaining 5 animals continued to show unusual fluctuations in temperature, but ultimately returned to normal temperature and vigor. These animals were bled from 1 to 2 months after the disappearance of the suggestive symptoms or temperature elevations, and neutralization tests were done. The serums of 4 of the 5 animals failed to neutralize small doses of the stock virus. The fifth animal, monkey J1-03, neutralized the virus on two separate occasions.

The nasal washings inoculated into this animal came from a 6 year old child, I.F., 43 days after the onset of illness. Since the virus was not actually isolated and subjected to passage, the presence of neutralizing substance in the serum of this monkey (J1-03) can be considered only as presumptive evidence of the presence of the poliomyelitis virus in the nasal secretion of this child. It is interesting to note that a second case appeared in this family. I.F., the child referred to above, became ill on Sept. 22, 1935, and within 3 days developed definite paralysis. A younger child (R.F., 4 years old) became ill with the disease on Oct. 11, 1935. Nasal washings taken from this second child yielded a negative result.

SUMMARY

The positive detection of the virus of poliomyelitis in the nasal secretions of 2 children, 16 and 13 days after the onset of the disease, is described. 7 animals which had been inoculated with other concentrates became ill with symptoms and temperature elevations suggestive of poliomyelitis, from 1 to 3 weeks following inoculation, but without definite paralysis. In 2 of these animals which were sacrificed, the histologic findings were compatible with the diagnosis of poliomyelitis but were not typical. Of the serums of the 5 remaining animals 4 failed to neutralize stock virus, whereas the serum of the fifth neutralized the virus on two different occasions. This serum was obtained from a monkey that had been inoculated with concentrated nasal secretions of a child 43 days after the onset of illness.

It is suggested that the present quarantine period of 3 weeks is compatible with the available data. It is further suggested that the methods of procedure described may be useful in similar investigations.

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THE EPIDEMIOLOGY OF LYMPHOCYTIC CHORIO-MENINGITIS IN WHITE MICE

By ERICH TRAUB, V.M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Certain phases of an epidemic in a mouse colony due to the virus of lymphocytic choriomeningitis have been described in previous papers (1, 2). The experiments presented here were undertaken in the attempt to trace the origin of the virus and to study the epidemiology of the disease in mice.

So far as we know, the disease had never before been reported in white mice. Since mice infected naturally with the virus often show no symptoms, the infection is not readily recognized unless intracerebral inoculations of material are made from diseased to healthy mice. Following the work of Theiler on yellow fever such transfers have been frequently made within recent years by investigators of virus diseases, and yet choriomeningitis virus has apparently not been encountered. It may be assumed, therefore, that the disease is not widespread in stocks of white mice. Rivers and Scott (3-5) have clearly shown that they obtained the virus from two human patients. One of them is a staff member of this Department who had been working with mice from the infected stock; the other had had no known contact with diseased white mice. Armstrong and Dickens (6) have also presented evidence suggesting that the virus affects human beings. Armstrong and Wooley (7) obtained the virus from a monkey injected with poliomyelitis virus. They found some of their stock monkeys to be immune to choriomeningitis, and they also detected neutralizing antibodies in the blood serum of a caretaker of the monkeys who had shown no symptoms suggestive of meningitis. Armstrong and Lillie (8), who first described the virus, were not certain whether it had originated in man or monkeys.¹

¹ At the time this paper was completed a paper by Findlay, Alcock, and Stern (*Lancet*, 1936, 230, 650) reports the detection of the virus in a mouse autopsied in

The Epidemiology in the Infected Stock

The mouse stock in which the disease occurred was built up from about 100 breeding mice acquired in 1922 from a dealer in Pennsylvania. Since then no new mice have been added to the colony. The disease was first recognized early in December, 1934. It is not known how long it had been present. When, in the course of experiments performed in 1933, material was frequently passed from mouse to mouse by intracerebral injection, the disease was not observed and presumably was not present.

It was estimated that from 40 to 50 per cent of the colony, which numbered about 2000 animals, had become infected when the disease was recognized. From a few of the uninfected mice a new stock was built up which will be referred to as the virus-free stock.

Some of the infected mice were kept in strict isolation in five cages for a study of the epidemic. No new mice were added and no virus was introduced. In each cage there were eight to ten female mice of different ages, most of them full grown and many pregnant, and one male. Young mice born in the cages were removed at the age of 4 to 5 weeks except those needed as replacements. At times the females of a litter were left in the cage for a longer time in order to obtain mice of different ages for the immunity tests. Some of the young mice removed were bled by heart puncture and their blood was tested for virus to ascertain that the disease was still present.

The mice in these five cages have been under observation for 9 months during which time the disease has persisted in all cages without change in its character. Symptoms were noted only in young (1 to 6 week old) mice, and some of these died. The rates of morbidity and mortality varied greatly in different litters. The mice of many litters never showed symptoms, while in other litters all, or almost all, appeared sick and some died.

In order to determine the rate of infection and the age at which infection occurred, mice of different ages were tested for immunity by intracerebral inoculation with 0.04 cc. of a virulent 5 per cent mouse brain suspension. The results are given in Table I. All mice older

1934, as well as in the mice from one out of fifteen stocks tested, and in two human patients.

than 4 weeks were solidly immune. A number of the younger mice that were not immune died in typical convulsions 2 to 5 days after the test inoculation, that is, after a much shorter incubation period than that in control mice from a virus-free stock (6 to 7 days). It is probable that these mice were already infected and that the injury of the inoculation caused the virus to become localized in their brains. Previous observations (1, 2) are in accord with this view.

The difference between the high rate of infection (100 per cent) among the mice of the five cages kept for a study of the epidemic and

TABLE I
Results of Immunity Tests in Mice of Different Ages from Diseased Stock

Age of mice	No. tested	No. immune
1-2 wks.	1	0
2 "	8	4
2-3 "	2	2
3 "	40	36
3-4 "	17	15
4 "	27	27
5 "	7	7
6 "	17	17
7 "	7	7
8 "	2	2
2-3 mos.	1	1
4 "	4	4
Over 5 "	14	14

Each mouse was given an intracerebral injection of 0.04 cc. of a 5 per cent suspension of brain from infected mice.

that in the infected colony (40 to 50 per cent) is accounted for by the fact that in the colony the disease was not present in all breeding cages.

The young from the colony were weaned at the age of 3 to 4 weeks and transferred to other cages in which they remained until they were given out to the laboratories. Each of these cages contained about 25 mice which came from different breeding cages. The mice from breeding cages in which the disease was not present became infected by contact with the mice from infected cages. This is the reason why in earlier experiments (1, 2) some 5 week old mice were encountered

which were not yet immune and showed characteristic symptoms following an intracerebral injection with sterile bouillon.

Experiments on the Mode of Transmission of the Disease

Immune Mice as Carriers of Virus.—As reported (9), some mice which recovered after natural or experimental infection remained carriers of the virus for several months. In other such mice the virus soon became undemonstrable. After it was established that practically all mice in the infected stock become infected when they are very young, it was of interest to determine approximately how many of them remained carriers. All mice examined appeared healthy.

The experimental methods used have already been described (9). Heart blood from each mouse was injected intracerebrally into a guinea pig, and the urine and nasal washings were injected subcutaneously into the footpads of guinea pigs. Nearly all guinea pigs used in these tests had been previously inoculated with sublethal doses of equine encephalomyelitis virus. It is not probable that these inoculations altered their susceptibility, since there is no cross-immunity between the two diseases.

Table II shows that several mice carried virus in the blood and discharged virus with the urine and nasal secretions. The duration of the carrier stage was estimated on the assumption that the infection of the animals occurred when they were less than 4 weeks of age. The number of carriers decreased with increasing age.

Intrauterine Infection.—An attempt was then made to determine whether the virus passed to the embryos from pregnant carriers.

In the 1st and 2nd experiments recorded in Table III the pregnant mice were killed by bleeding from the heart under deep ether anesthesia. The embryos were removed aseptically, immersed in absolute alcohol for about 10 minutes, and then in acetone for about 2 minutes. The defibrinated blood and a 20 per cent suspension of the entire embryos were titrated, and the decimal dilutions were injected either subcutaneously into the plantae of guinea pigs or intracerebrally into mice from a stock free from the disease. (This stock was acquired from a dealer (Freed) who also supplied some of the mice used by Rivers and Scott.) In other experiments indicated in Table III the mother mice were bled shortly after parturition, and the blood as well as suspensions of the brains of the new born young was tested for virus. The young mice were chloroformed and then washed in alcohol and immersed in acetone before their brains were removed. The detection of virus in the

brains of new born mice is regarded as an indication of intrauterine infection, because the time elapsing between birth and the test was too short for contact infection. The details of the experiments are recorded in Table III.

In the 1st and 2nd experiments the virus content of the embryo suspensions was higher than that of the blood of the mother mouse, a fact which indicates that the virus multiplied in the embryos. Intrauterine infection of the embryos occurred in four out of six naturally infected pregnant mice which carried virus in the blood. In the mouse tested in Experiment 6 no virus had been detected in the blood 2 weeks before parturition, but at autopsy performed 6 hours after parturition a lymphosarcomatous mediastinal tumor was discovered

TABLE II
Virus Carriers in Diseased Stock

Approximate age of mice	Tests for virus in								
	Blood			Urine			Nasal washings		
	No. tested	No. positive	No. negative	No. tested	No. positive	No. negative	No. tested	No. positive	No. negative
2-3 mos.	3	3	0	2	2	0	2	2	0
About 4 " *	9	7	2	3	3	0	3	1	2
4-5 "	4	4	0	4	3	1	4	4	0
Over 5 "	10	3	7	3	1	2	3	1	2

* In one mouse of this group virus was detected in the urine but not in the blood or nasal secretions.

which contained virus. Experiment 8 was made with four experimentally infected mice from the virus-free stock. The result suggests that the passage of the virus through the placenta requires a considerable time.

The rate of morbidity in litters infected *in utero* is usually high, often amounting to 100 per cent. The mortality varies between 0 and 60 per cent. The disease is chronic in these mice, and the symptoms, which begin about 1 week after birth and may last for over a month, are a greatly decreased rate of growth, emaciation, slow and stiff movements, slight somnolence, a ruffled fur, and sometimes diarrhea.

TABLE III

Intrauterine Transmission of Virus in Mice

Experiment No.	No. of mice examined	Mode of infection of mother mice	Material	Approximate age of embryos or young	Test animals	Tests for virus in			
						Heart blood of mother mouse		Suspension of embryos or brains of new born young	
						Undiluted	Titer	Undiluted	Titer
1	1	Natural	E	2 wks.	Guinea pigs	+	10 ⁻⁴	+	10 ⁻⁵
2	1	"	"	2 "	Mice	+	10 ⁻¹	+	10 ⁻⁶
3	1	"	Y	Less than 4 hrs.	Guinea pigs	+	—	+	10 ⁻⁴
4	1	"	"	" 4 "	"	+	—	+	—
5	2	"	E	2-3 wks.	"	+	—	+	—
6	1*	"	Y	6 hrs.	"	+	—	+	—
7	6	"	E	2 wks.	Mice	+	10 ⁻¹	0	0
			Y	2-24 hrs.	"	+	10 ⁻²	0	0
			E	2 wks.	"	+	—	0	0
8	1 (sick)	Intracerebral inoculation 7 days previously	"	2 "	Guinea pigs	+	—	+	<10 ⁻⁴
	1 (sick)	"	Y	A few hrs.	"	+	—	+	+
	1 (sick)	Intravenous inoculation 4 days previously	E	2 wks.	"	+	—	+	+
	1 (sick)	Intravenous inoculation 10 days previously	E		"	+	—	+	+

+ = virus detected; 0 = no virus detected; — = titer not determined.

E = embryos; Y = new born young.

* In this mouse virus was detected in a lymphosarcomatous mediastinal tumor.

Several mice which had become infected *in utero* were born dead, but abortion has not been noted in pregnant carrier mice.

Infection by Contact with Diseased Mice.—Since all mice from the infected stock more than 3 to 4 weeks old were immune, it seemed possible that infection by contact took place in those mice not infected *in utero*. It was known that infected mice discharge virus with the urine and nasal secretions.

Eight experiments on contact infection were carried out with mice whose ancestors had been free from the disease for at least 2 or 3 generations.

From each mouse to be exposed 0.2 cc. blood was drawn by heart puncture. The blood samples were pooled and defibrinated, and the pooled blood was injected into a guinea pig (0.2 cc. intracerebrally, the remainder subcutaneously into the plantae), with a negative result in all experiments. The litter mates of these mice not used in the experiments were tested for immunity by intracerebral inoculation with virus as additional controls for the exposed mice. They all died of typical choriomeningitis. The ages of the exposed mice varied from 3 weeks to 3 months, and in two experiments mice with newly born litters were included. The mice were exposed by placing each lot in a separate cage with a number of mice which had just been inoculated. The routes of inoculation are indicated in Table IV. Precautions were taken that the mice which died were not devoured by the other mice.

Two methods were used to determine whether or not the exposed mice had become infected: They were bled again 3 to 4 weeks after the 1st day of exposure, and their pooled blood was injected into a guinea pig as above. If this test was negative, it was repeated 8 days later. The mice which survived the bleedings were tested for immunity by intracerebral injection with 0.04 cc. of a 5 per cent mouse brain suspension 4 to 5 weeks after the 1st day of exposure. The percentage of infected mice was based upon the latter tests.

Table IV shows that the virus is readily transmitted by contact provided the time of exposure is sufficiently long. None of the mice infected by contact showed symptoms.

Another experiment was carried out with a litter of nine mice born in the infected stock from a mother with avirulent blood. Five of the young were killed about 12 hours after birth and no virus was detected in their brains. The remaining young were left with the mother and other mice of the same stock. On the 4th day after birth two were killed and virus was present in their brains. The remaining two

never showed symptoms and were later resistant to intracerebral injection with virus. From this experiment it seems that in new born mice contact infection takes place more rapidly than in older mice.

Several other litters born in infected cages from mice with virus-free blood were observed for about 2 months for clinical symptoms but

TABLE IV
Experiments on Contact Infection in Mice

TABLE IV									
Experiments on Contact Infection in Mice									
Experiment No.	Injected mice					Length of time mice were in contact	Exposed mice		
	No.	Route of injection	Result				No.	Result	
			Died	Became sick and re-covered	Showed no symptoms			Blood test (3-4 wks. after 1st day of exposure)	Percentage of mice that became immune
1	4	Intracerebral	4	0	0	7	3	-	0
2	4	"	4	0	0	7	3	-	0
3	6	"	6	0	0	8	2	-	0
4	10	"	10	0	0	10	2	+	100
5	6	"	3	3	0	17	7	+	100
							plus 18 new born young		
6	6	"	5	1	0	34	2	-	100
7	5	Intravenous	0	2	3	21	6	+	100
							plus 3 new born young		
8	6	3 intravenous 3 intracerebral	0 1	3 2	0 0	25	6	+	50
									75

detected in pooled blood; + = virus detected in pooled blood.

did become infected

— = no virus detected in pooled blood; + = virus detected in pooled blood.

none were noted, although the fact that the mice did become infected by contact is evidenced by their immunity to a later intracerebral test inoculation.

A few experiments analogous to the ones described above were carried out with white mice from the Freed stock² in which neither carriers nor immune animals had been detected. The mice were

² These are Swiss mice from a dealer who also supplied Dr. Rivers and Dr. Scott.

highly and uniformly susceptible to intracerebral injection with virus. In these experiments virus was never demonstrated in the blood of the 37 mice exposed for 3 to 4 weeks to mice of the same strain injected intraperitoneally with virus, and all exposed mice died following the intracerebral test injection.

In another experiment ten mice from the virus-free stock were exposed together with ten mice from the Freed stock. The mice were bled after the exposure and no virus was present in the pooled blood samples from either group. However, all the mice of the first group that survived the bleedings became immune, while of the second group only two of nine mice were immunized.

The Portal of Entry in Mice.—Preliminary experiments on the mode of infection gave the following results: (a) Virus is discharged from acutely ill mice and immune carriers with the urine and nasal secretions (2, 9). (b) Mice failed to become infected by feeding with infected mouse brain suspension, or by repeated feeding with virulent mouse urine given on bread. (c) The dropping of virus into the conjunctival sac also failed to infect mice. (d) Virus instilled into the nares, however, infected a certain percentage of the mice which did not show symptoms as did those infected by contact (2). This evidence pointed to the nose as the portal of entry of the virus.

In each experiment recorded in Table V, five 3 week old white mice from the virus-free stock were placed in the same cage with a naturally infected mouse. In Experiment 1 the mice were placed in contact with the carrier mouse 15 described previously (9) 184 days after this animal was removed from the infected stock. In a preliminary experiment this mouse had transmitted the disease to two healthy mice. Mouse 15 discharged virus through the nose and urine during the present experiment (tests recorded in Table V). In Experiment 2 a mouse was used which had carried and discharged virus for at least 4 months. Virus was present in the nasal washings taken from this animal shortly before it was placed in the same cage with the young mice. The urine was not tested for virus. The cages used in the experiments had screen bottoms to eliminate infection through urine as far as possible. The exposed mice were bled daily by heart puncture, 0.15 cc. blood being withdrawn from each mouse. Immediately after the bleeding their pooled, defibrinated blood was injected into a guinea pig (0.2 cc. intracerebrally, and the remainder subcutaneously into the plantae). Several mice died from injury through the heart puncture (see Table V). Nasal washings also were taken daily from the exposed mice according to the method described previously (9), and the pooled nasal washings were injected subcutaneously into the plantae of a guinea pig.

If virus entered the exposed mice by way of the nose, it should be detected in the nasal washings before it appeared in the blood. This was the case in Experiment 1. The two mice which survived the

TABLE V
Portal of Entry of Virus in White Mice

TABLE V								
Portal of Entry of Virus in White Mice								
Day of exposure	Experiment 1					Experiment 2		
	Tests for virus in infected mouse 15		Tests for virus in 5 mice placed in contact with mouse 15		Accidental deaths	Tests for virus in 5 mice exposed to an infected mouse		Accidental deaths
	Urine	Nasal washings	Nasal washings	Heart blood		Nasal washings	Heart blood	
Shortly before exposure	+	+	—	0		0	0	
2	—	—	0	0		—	—	1
3	—	—	0	0		0	0	
4	—	—	0	0		+	0	1
5	—	—	0	0		0	0	
6	—	—	0	0		0	0	
7	—	—	0	0	2	0	0	
8	+	+	+	0		+	0	
9	—	—	0	0		+	0	2
10	—	—	0	0		+	0	
11	—	—	+	+		+	0	
12	—	—	+	+	1	+	—	
13	—	+	+	+		—		
14	+	+	—	1 mouse +† 1 " 0†		—	0†	
9 days after removal from infected mouse*								
10 days after removal from infected mouse								
† = not for a guinea pig; 0 = avirulent for a guinea pig; — = no test								
* = removed from the infected mouse								

+ = virulent for a guinea pig; 0 = avirulent for a guinea pig; — = no test made.

* In both experiments the contact mice were removed from the infected mouse on the 14th day.

† Both surviving mice were tested for immunity on this day and were immune.

‡ The surviving mouse was tested for immunity on this day and was not immune.

bleedings were later immune to intracerebral injection with virus. In Experiment 2 virus was repeatedly detected in the nasal washings from the exposed mice but never in the blood. One mouse survived the

bleedings. In its nasal secretions virus was present on at least 2 successive days, but the animal did not become generally infected or immune.

The occurrence of contact infection in spite of the fact that the animals were kept on screens suggests that the nasal secretions of infected mice are more important in the transmission of the virus than the urine. The negative results obtained in two experiments in which an attempt was made to infect mice by placing them in cages heavily contaminated with virulent urine support this assumption. Additional evidence for it is the failure of the white mice (in Experiments 2 and 3, Table V) to become infected when kept in the same cage with infected wild mice whose urine was virulent. White mice and wild mice, when kept in the same cage, keep apart from each other during the first 3 or 4 weeks and their noses rarely come in contact. This may be the reason why the white mice failed to become infected.

The Experimental Disease in the Wild House Mouse (Mus musculus)

Since it was possible that our mouse colony had become infected through contact with wild house mice, many wild mice were trapped in the stables and barns of the Institute and their blood was tested for virus.

The blood was drawn by heart puncture (0.3 cc. per mouse) from 102 mice. The mice were divided in lots of three to twelve animals. The blood samples from each lot were pooled and defibrinated and then injected into a guinea pig (0.2 cc. intracerebrally, 1 cc. into each planta, and the remainder intraperitoneally, if there was a large amount of blood). No virus was detected in any of the blood samples. Some of the mice which survived the heart puncture were used in experiments on contact infection immediately after the bleeding.

Another group of 45 wild mice was tested for immunity by intracerebral injection with 0.04 cc. of a virulent 5 per cent mouse brain suspension. Of these mice 40 died in 6 to 9 days or were killed when presenting typical tremors and clonic-tonic convulsions. Five mice showed no symptoms. It is not certain whether they contracted a subclinical infection, or were specifically immune, or naturally resistant. Blood drawn from four of them 1 month after the injection contained virus. In another experiment three wild mice were injected intra-

peritoneally with virus. They showed no symptoms. Their immunity was tested by intracerebral injection with virus given 38 days later. Pooled serum obtained from these mice 78 days after the test of immunity contained virus. These results indicate that the virus may persist after recovery in wild mice also and render the absence of virus from the blood of the 102 mice all the more significant.

Contact Infection in Wild Mice.—For epidemiological reasons it was of interest to determine whether wild mice may become infected by contact.

In a preliminary experiment it was found that wild mice injected intraperitoneally with virus discharged it with the nasal secretions and urine on the 8th day following the inoculation. No tests were made on other days. In Experiment 4 (Table VI) the urine and nasal washings from the wild mice on the 8th day after the inoculation also contained virus.

Four experiments on contact infection were made. The method used was essentially the same as that in the experiments on contact infection with white mice.

In Experiments 2, 3, and 4 (Table VI) some white mice bred from virus-free parents were exposed to the infected mice together with the wild ones. The shavings used as bedding in the cages were not changed until the first bleeding, in which 0.2 cc. blood was drawn by heart puncture from each mouse. The pooled, defibrinated blood was injected into a guinea pig (0.2 cc. intracerebrally and the remainder subcutaneously into the plantae). The exposed mice were in contact with the infected ones until the intracerebral test inoculation (0.04 cc. virulent 1 per cent mouse brain suspension) which was administered on the 30th to 38th day of exposure. All exposed mice, the white and wild ones alike, showed no symptoms before the test injection.

Table VI gives the details of the experiments. No transmission of the virus occurred in the 1st and 2nd experiments, while in the 3rd experiment the virus passed to the exposed wild mice, as indicated by the positive blood test, but not to the white ones. All the surviving exposed mice died following the test of immunity. Since a general infection with choriomeningitis produces a solid immunity, it must be assumed that the wild mice infected by contact were among those which died from injury either following the heart puncture or the intracerebral test inoculation. In the 4th experiment all of the wild

TABLE VI
Experiments on Contact Infection in Wild Mice

Experiment No.	Injected mice			Exposed mice									
	No. and variety	Route of inoculation	Result	Blood tests						Test for immunity by intracerebral inoculation with virus			
				1			2			3			
				Day of exposure	Result	No. dying from injury	Day of exposure	Result	No. dying from injury	Day of exposure	Result	No. dying from injury	No. resistant
1	7 wild 4 "	ip ic	1 D, 6 S 3 D, 1 S	18	—	3				34	1	0	0
2	9 "	ip	2 D, 7 S	17	—	0	23	—	2	28	—	0	0
3	6 "	ip	4 D, 2 S	18	—	2	23	—	0	28	—	0	0
4	3 " 3 "	inas ip	3 S 1 D, 2 S	19	+	1	25	—	0	30	4	0	0
	3 white 3 "	inas ip	3 S (sick) 3 S	19	+	1				30	0	0	0
										38	2	7	0
										28	1	5	0

In Experiments 1, 2, and 3 the injected mice and exposed ones were placed in the same cage immediately after the inoculation. In Experiment 4 the mice were put together on the 3rd day after the injection.

ip = intraperitoneally, ic = intracerebrally, inas = intranasally.

D = died; S = survived; — = no virus detected; + = virus present.

mice and all of the white mice became infected as shown by the positive blood tests and the immunity acquired by the mice.

The Disease in Guinea Pigs

Late in January and in February, 1935, three guinea pigs injected with equine encephalomyelitis virus developed choriomeningitis. This virus was recovered from all three, and each strain was pathologically and immunologically identical with the virus obtained from mice. Although proof is lacking, it seems probable that the animals did not become infected through the inoculations.

The guinea pigs were kept in different cages but in the same unit with other guinea pigs and about 6 feet away from several cages of mice from the infected stock. The disease has not been observed in any other guinea pigs in our stock, and every stock guinea pig tested thus far has been susceptible, indicating that the three infections described were exceptional.

Infection by Contact in Guinea Pigs

Of thirteen guinea pigs placed in cages with diseased ones, one contracted the disease. Transmission of the virus by contact occurred accidentally on two occasions when guinea pigs injected with innocuous material were kept in the cages with infected guinea pigs.

The disease in our guinea pigs is not so highly contagious as in our white mice. Prolonged contact appears to favor the transmission of the virus as in mice.

Neutralization Tests with Human Sera

It seemed possible that the mouse colony and three guinea pigs just described had become infected in the unit through contact with the caretakers. The sera of the three caretakers of this unit, one of whom (J. M.) had also taken care of the infected mouse colony for the past 5 years, were tested for antiviral. These neutralization tests were made by the method already described (8), by which mixtures of decimal dilutions of virus and equal amounts of undiluted serum were injected subcutaneously into the plantae of guinea pigs.

In Table VII the results of the two tests made with J. M.'s serum are given. It neutralized about 100 M.I.D. (minimal infective doses) of virus.

In Table VIII neutralization tests are recorded with three samples of serum drawn at different times from J. S., a caretaker of the infected guinea pigs. It is noteworthy that the serum drawn on March 3, 1935,

TABLE VII
Neutralization Tests with Serum of J. M.

Virus dilution	First test						Second test	
	J.M. serum Feb. 4, 1935	Normal human sera (controls)			Normal guinea pig serum (control)	Immune guinea pig serum (control)	J.M. serum Feb. 4, 1935	Normal human serum B.T. (control)
		B.T.	E.T.	G.J.				
10 ⁻¹	D 15	D 14	D 13	D 13	D 14	D 22	—	—
10 ⁻²	—	—	—	—	—	—	D 16	D 16
10 ⁻³	0	D 15	D 22	D 17	D 15	0	0	D 16
10 ⁻⁴	—	—	—	—	D 14	—	0	D 16
10 ⁻⁵	—	—	—	—	D 17	—	0	F, no S
10 ⁻⁶	—	—	—	—	—	—	0	0

D 15 = died in 15 days; F = fever; S = typical symptoms followed by recovery; 0 = no fever, no symptoms; — = not tested.

TABLE VIII
Neutralization Tests with Serum of J. S.

Virus dilution	First test					Second test			Third test	
	Serum of J.S. Mar. 5, 1935	Human control sera				J.S. Mar. 5, 1935	J.S. July 20, 1935	G.J. (control) Feb. 4, 1935	J.S. Sept. 16, 1935	G.J. (control) Feb. 4, 1935
		E.T. Feb. 4, 1935	G.J. Feb. 4, 1935	B. Mar. 5, 1935	E.B. Mar. 5, 1935					
10 ⁻¹	D 18	D 13	D 13	D 12	D 14	D 24	D 21	Severe S	—	—
10 ⁻²	D 18	D 16	D 17	D 4*	D 14	D 19	0	D 17	0	D 14
10 ⁻³	0	D 17	D 17	D 16	D 18	D 24	0	D 17	0	Severe S
10 ⁻⁴	0	F, no S	0	0	0	Severe S	0	Severe S	0	D 15
10 ⁻⁵	0	0	0	0	0	—	—	0	0	D 29
10 ⁻⁶	—	—	—	—	—	—	—	—	0	0

D 18 = died in 18 days; F = fever; S = typical symptoms followed by recovery; 0 = no fever, no symptoms; — = not tested.

* Died of intercurrent disease.

had very little neutralizing power, while the serum drawn on July 20 neutralized 100, and on September 16 at least 1000 M.I.D. of virus.

Neither J. M. nor J. S. at any time showed symptoms suggestive of meningitis. If they were infected, their disease was subclinical. The serum of the third caretaker did not neutralize the virus.

DISCUSSION

Since symptoms were noted in white mice that had become infected *in utero*, while other mice born virus-free but infected soon after birth by contact with diseased mice showed no symptoms, it seems that the virus affects embryonic mouse tissues more severely than mature tissues.

Immune carriers probably are in part responsible for the persistence of the disease in the mouse stock for at least 15 months. Another factor favoring this long persistence is the absence or exceedingly low concentration of antiviral in the blood of immune mice (9). In other virus diseases the antiviral is known to be transmitted by immune mothers to the suckling young, which during their passive immunity either fail to become infected or acquire a lasting active immunity due to a mild infection under the partial protection of the antiviral. But young mice born from immune, virus-free mothers are in no way protected from choriomeningitis. They become infected a few days after they are born.

As shown by the different results obtained with mice descended from the infected colony and the mice obtained from a dealer (Freed), white mice from different stocks are not equally susceptible to contact infection with the virus under study. It would be of interest to determine whether hereditary factors are responsible for this difference. However, the virus is difficult to work with experimentally from this viewpoint, because the mice infected by contact show no symptoms.

It is possible that the two caretakers whose sera neutralized the virus had had a subclinical infection. The increase of the neutralizing power of J. S.'s serum between March 3 and September 16, 1935, is in favor of the specificity of the neutralizing effect of this serum. Since antiviral develops slowly in man, according to the evidence of Scott and Rivers (4), J. S. may have become infected at about the same time or shortly before the guinea pigs which he took care of were infected. No evidence was obtained as to the time of the hypothetical infection of J. M.

The question whether or not an infected human being can transmit the disease directly to lower animals cannot be answered at present. The transmission of the virus from man to guinea pigs is doubtful since these animals rarely become infected by contact with infected guinea

pigs which discharge considerable amounts of virus with the nasal secretions and urine. With old mice it is also doubtful, because they require a rather close contact with infected mice over a relatively long time to become infected. In new born mice, however, contact infection occurs more rapidly and probably more easily than in older ones, and it may be supposed that the virus gained a foothold in such mice, if the caretaker was the source of infection for the mouse stock.

On the other hand, it is possible that the caretakers became infected through contact with the diseased animals. As Rivers and Scott (5) pointed out, however, the virus is not readily transmitted from lower animals to man. Two workers in our laboratory have handled infected mice or guinea pigs almost daily for 15 months without developing any antiviral.

SUMMARY

In a mouse colony in which lymphocytic choriomeningitis is endemic infection takes place either *in utero* or shortly after birth. Virus is discharged from infected mice with the nasal secretions and urine. In some mice the infection lasts for several months, and such carriers can transmit the disease to healthy mice by contact. The portal of entry appears to be the nasal mucosa rather than the gastrointestinal tract. Mice infected by contact show no definite symptoms while those infected *in utero* often do. The disease has persisted in the colony for at least 15 months without change in its character. Mouse stocks differ in their susceptibility to contact infection and the findings given in the paper could be reproduced only with a very susceptible stock. Wild mice (*Mus musculus*) can be infected by contact, although less easily than our white mice.

The source of the infection in the colony has not been determined. The fact that the serum of the caretaker neutralizes the virus indicates that he has been infected. It seems likely that the virus went from him to the mice rather than *vice versa*. Other possible sources of infection are considered.

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THE COMPLEMENT FIXATION REACTION WITH PNEUMOCOCCUS CAPSULAR POLYSACCHARIDE

By KENNETH GOODNER, PH.D., AND FRANK L. HORSFALL, JR., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The extreme sensitivity of the complement fixation reaction as usually applied in immunology is well recognized. However, certain unique findings have been reported with the use of bacterial polysaccharides as antigens. For example, Zinsser and Parker (1) found that no fixation could be obtained with combinations of pneumococcus capsular polysaccharide and specific immune horse sera, although complement was bound when the same substances were combined with specific immune rabbit sera. Similar results were reported by Pittman and Goodner for the specific polysaccharide of *Hemophilus influenzae*, Type b (2).

The present experiments constitute a critical survey of this subject with especial regard to the general mechanism of the complement fixation reaction. It must be appreciated that the use of purified specific polysaccharides offers some advantage in that the quantity and character of at least one reagent in this most complicated of tests can be carefully controlled.

Materials and Methods

Complement Fixation Test.—The reagents used were: fresh guinea pig serum (complement), a 5 per cent suspension of washed sheep red blood cells, and the serum of a rabbit immunized with sheep red blood cells (amboceptor). Each reagent was used in a volume of 0.5 cc. The amboceptor was titrated with excess of complement and so diluted that 0.5 cc. contained 2 units. The complement was titrated against 2 units of amboceptor. The final volume in each tube of the titrations was 1.5 cc. Further details of the methods used in the various experiments will be presented with the respective protocols.

The majority of these experiments have been carried out with the capsular polysaccharide (acetyl form) of Type I Pneumococcus. Type-specific anti-pneumococcus horse and rabbit sera have possessed equivalent agglutinin and precipitin titers.

The Sensitivity of the Complement Fixation Test

In order to establish the essential sensitivity of the reaction, the results of an experiment, which consists of a comparison between precipitin and complement fixation titers of Type I antipneumococcus rabbit serum, are reproduced in Table I. It will be noted that, as the amount of immune serum was decreased, smaller amounts of polysaccharide were required in order to obtain maximum reactions. In our hands the limit of the reaction with complement fixation was reached

TABLE I
Comparison of Precipitin and Complement Fixation Titers of Type I Antipneumococcus Rabbit Serum

Dilution of acetyl polysaccharide	Dilution of immune rabbit serum					
	1:2		1:10		1:50	
	Precipitin reaction	Complement fixation	Precipitin reaction	Complement fixation	Precipitin reaction	Complement fixation
1:2,500	++++	+++++	—	+++	—	—
1:12,500	++++	+++++	+	+++++	—	—
1:62,500	++++	+++++	+++	+++++	±	+
1:312,500	++++±	+++++	++	+++++	—	++++±
1:1,562,500	+	+++++	+	+++++	—	++++
1:7,812,500	—	+++	—	+++	—	+++++
1:39,062,500	—	+	—	—	—	±

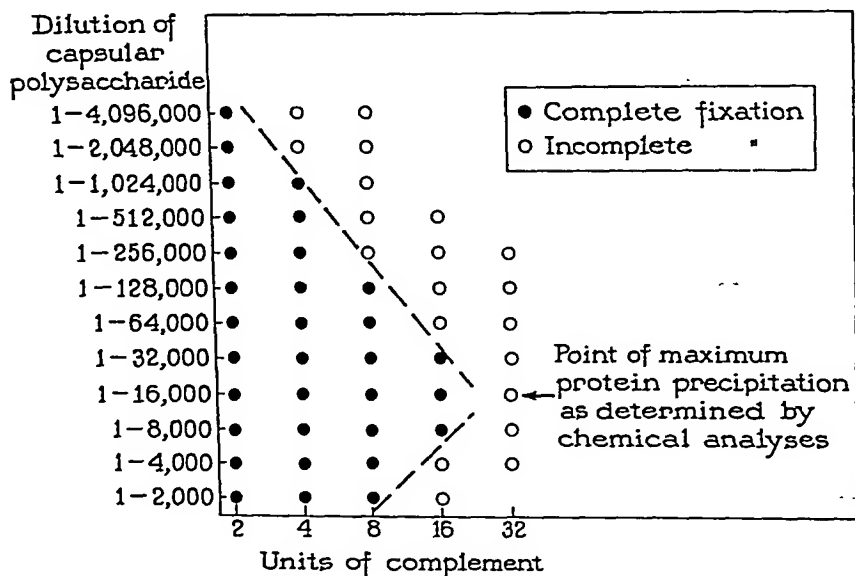
with serum diluted 1:200 and specific polysaccharide diluted 1:20,000,000. It may be pointed out that in many instances combinations gave complete fixation in the absence of any visible precipitation.

Quantitative Complement Fixation

In an experiment the results of which are plotted in Text-fig. 1, the maximum number of units of complement fixed by given combinations of polysaccharide and immune rabbit serum was determined. It will be noted that, with certain amounts of the polysaccharide, the amount of complement which was fixed was large, that is, at least 16 units. This point of maximum fixation is also the point of maximum protein precipitation as determined in a parallel experiment carried out by the method of Heidelberger, Sia, and Kendall (3).

Thus the results suggest that, within certain limits, the property to fix complement is proportional to the quantity of immune precipitate formed in the same reaction.

As a means of testing this hypothesis, quantitative fixation tests were carried out with three forms of Type I pneumococcus capsular polysaccharide: (a) the viscous product described by Heidelberger and Kendall (4), (b) the acetyl polysaccharide of Avery and Goebel (5), a product which may be derived from the first mentioned form by



TEXT-FIG 1. The number of units of complement fixed by varying amounts of pneumococcus capsular polysaccharide in combination with specific immune rabbit serum diluted 1:4.

means of heat, and finally, (c) the deacetylated product which results when the acetyl form is treated with alkali (5). As shown by Heidelberger and Kendall (4), the capacities of these three forms of the polysaccharide to precipitate immune globulin from specific antipneumococcus rabbit serum are in this same order.

The results of the quantitative complement fixation test are shown in Table II. It will be noted that with the deacetylated form only 2 units of complement were fixed, with the acetyl form 16 units were fixed, while the viscous polysaccharide

completely fixed 32 units of complement. The maximum amounts of protein specifically precipitable by these three forms of polysaccharide are also shown in Table II.

These results are a convincing demonstration that under these conditions the amount of complement which can be fixed is proportional to the amount of immune precipitate formed.

If one conceives of the precipitin reaction as at least diphasic, that is, consisting of a stage of specific union of antigen and antibody fol-

TABLE II

Amount of Complement Fixed by Combinations of Immune Rabbit Serum and Various Forms of Type I Capsular Polysaccharide

Type I polysaccharide	Specifically precipitable protein per cc. of immune serum	Units of complement					
		2	4	8	16	32	64
	mg.						
Deacetylated polysaccharide.....	1.595	++++	++±	+	—	—	—
Acetyl polysaccharide..	4.139	++++	++++	++++	++++	+++	—
Viscous acetyl polysaccharide of Heidelberg and Kendall..	4.640	++++	++++	++++	++++	++++	++±

The complement fixation reactions were carried out with immune rabbit serum diluted 1:10 and the various polysaccharides diluted 1:100,000. Complement was added immediately after admixture of antiserum and polysaccharide. Precipitable protein determinations were carried out after the method of Heidelberger, Sia, and Kendall (3).

lowed by a second stage of flocculation, there arises the question of the temporal position of complement fixation in this series of reactions.

As an approach to this problem, quantitative fixation tests were carried out with mixtures which differed only in the time which elapsed before the addition of complement. The results of one experiment are shown in Table III. It will be noted that, in instances in which the addition of complement was made an hour or more after preliminary incubation of polysaccharide and specific antibody, only 2 units were completely fixed. When, however, the preliminary incubation had

occupied only 10 minutes, 8 units were completely fixed. When complement was present at the time of admixture of antigen and antibody, 32 units were fixed.

With preliminary incubations of 1 hour or longer, flocculation had occurred; in the instance of the 10 minute period no flocculation was apparent when complement was added. It is obvious that under these circumstances the total surface exposure of immune aggregates had diminished with the time of incubation, for the surface presented by large flocs is much less than that which would be presented by all of

TABLE III

Amount of Complement Fixed with Reference to the Time of Addition of this Reagent

Conditions	Units of complement				
	2	4	8	16	32
A. Polysaccharide + serum 4 hrs. 37° then complement added	++++	++++±	+	—	—
B. Polysaccharide + serum 1 hr. 37° then complement added	++++	++++±	++	—	—
C. Polysaccharide + serum 10 min. 37° then complement added	++++	++++	++++	+++	—
D. Complement added before admix- ture of polysaccharide and im- mune serum	++++	++++	++++	++++	++++

In this experiment all tubes contained equal amounts of Type I antipneumococcus rabbit serum diluted 1:10 and the acetyl polysaccharide diluted 1:100,000.

their component particles, were these evenly suspended. It would appear, therefore, that the amount of complement which can be fixed is a function of the total surface exposure of the immune aggregates.

As a further demonstration of this point, advantage was taken of the fact that, within certain limits, flocculation is more rapid the smaller the diameter of the tube. Quantitative fixation tests were carried out in tubes of three different diameters and the results are shown in Table IV. It will be noted that in tubes having a diameter of 0.81 cm. only 4 units of complement were fixed, while in tubes of 2.40 cm. 16 units were completely fixed. These results can be explained only by the fact that in smaller tubes flocculation is rapid and consequently the time-surface factor is greatly reduced.

A third direct demonstration of this surface phenomenon was obtained by centrifugation. Mixtures of polysaccharide and antibody which had been incubated 1 hour were whirled in the angle centrifuge at high speed for 10 minutes. Complement fixation tests were then carried out on both the clear supernatant fluid and the resuspended precipitates.

TABLE IV

The Amount of Complement Fixed with Reference to the Speed of Flocculation as Conditioned by Tube Diameter

Tube diameter	Mean flocculation time	Units of complement					
		2	4	8	16	32	64
cm.	min.						
0.81	21	++++	++++	±	—	—	—
1.26	33	++++	++++	++++	+++	++	—
2.40	43	++++	++++	++++	++++	+++	±

All tubes contained equal amounts of specific immune rabbit serum diluted 1:10 and acetyl polysaccharide diluted 1:100,000.

TABLE V

Complement Fixation after Centrifugation

Acetyl polysaccharide	Whole mixture not centrifuged	Precipitate	Supernatant
1:8,000	++++	—	++++
1:32,000	++++	—	++++
1:256,000	++++	—	+++±
1:1,024,000	++++	—	+++

Immune rabbit serum diluted 1:4. Incubated with polysaccharide 1 hour at 37°C. One set of tubes then centrifuged 10 minutes and the sediment and supernatant separated. Sediments thoroughly resuspended in saline. 2 units of complement added to each tube.

The results of one of these experiments are shown in Table V. It will be noted that the uncentrifuged mixture gave complete fixation with all dilutions of the polysaccharide. None of the resuspended precipitates gave any fixation. On the other hand, the property of fixation remained in the supernatant fluid, although in somewhat reduced amount. In other experiments in which *quantitative* fixation was carried out, this decreased capacity for fixation was clearly demonstrated. Thus a combination capable of fixing several units of complement lost a

large proportion of this capacity after 10 minute centrifugation, and all of it after 30 minutes at very high speed. In any instance in which centrifuged combinations possessed the capacity of fixation this property resided in the clear supernatant fluid and not in the precipitate.

It may be pointed out that centrifugation speeds up the flocculation or secondary phase of the precipitin reaction, and in this sense greatly reduces the time-surface factor essential to the property of fixation.

Another question which remains to be answered: Is complement fixed by immune combinations in true solution, or rather by aggregates in a finely dispersed colloidal state? As an approach to this problem, experiments were carried out with certain "synthetic" antigens and the corresponding antisera.¹ This system consisted of the following reagents: (a) aminobenzyl glucoside, (b) the same coupled to chicken serum protein, and (c) serum of rabbit immunized to (a) coupled with the proteins of horse serum. The azobenzyl-gluco-chicken serum antigen has the property of reacting in precipitin tests with the immune rabbit serum. This serum, however, gives no visible reaction with the uncoupled glucoside. If, however, the glucoside is added to the immune serum, the latter loses the capacity of reacting with the azobenzyl-gluco-chicken serum antigen, that is, the uncombined glucoside has the capacity of combining with the antibody even though no precipitation occurs. (For the details of this phenomenon see Avery and Goebel (6).)

It has been found that complement is fixed only by the combination of immune serum and the azobenzyl-glucose-chicken serum antigen, not with the uncoupled glucoside. The evidence of the nature of this combination of uncoupled glucoside and immune serum is not sufficient to permit an exact interpretation of the findings with complement fixation, but the results suggest that the soluble immune combinations are incapable of fixing complement.

In so far as can now be determined, therefore, complement fixation is a phenomenon associated with particulation. It appears to be a matter of selective adsorption.

Inhibition of Complement Fixation by Immune Horse Serum

Zinsser and Parker (1) not only showed that immune horse serum gave no fixation of complement with specific bacterial polysaccharides,

¹ Furnished through the courtesy of Dr. Walther F. Goebel.

COMPLEMENT FIXATION

but they also demonstrated that immune horse serum would completely block the fixation normally obtained with immune rabbit serum.

In order to illustrate the type of inhibition by immune horse serum, a protocol of an experiment essentially similar to those reported by Zinsser and Parker (1) is shown in Table VI. The capsular polysaccharide of Type I Pneumococcus and the specific immune rabbit serum were allowed to react for 30 minutes. Dilutions

TABLE VI
Inhibition of Complement Fixation by Specific Immune Horse Serum

Tube	Type I pneumococcus capsular polysaccharide (acetyl form)	Type I anti-pneumococcus rabbit serum	Type I anti-pneumococcus horse serum					Result (fixation)
	0.5 cc.	0.5 cc.	0.5 cc.	10 minutes 37°C.	2 units complement added to each tube	30 minutes 37°C.	1 cc. sensitized sheep cells	
1	1:100,000	1:5	—	30 minutes 37°C.	10 minutes 37°C.	2 units complement added to each tube	30 minutes 37°C.	++++
2	"	1:25	—					++++
3	"	1:5	1:2					—
4	"	"	1:10					+++
5	"	"	1:25					—
6	"	1:25	1:2					—
7	"	"	1:10					—
8	"	"	1:25					—
9	"	—	1:2					—
10	"	—	1:10					—
11	"	—	1:25					—

Final volume in each tube, 3 cc.

In this, as in all subsequent experiments, suitable controls showed that none of the reagents were anticomplementary in the amounts used.

of Type I antipneumococcus horse serum were then added, followed by a second incubation of 10 minutes; guinea pig complement was then added and a third incubation of 30 minutes was carried out. At the end of this time sensitized sheep cells were added and the tubes were incubated again for 30 minutes.

From the results shown in Table VI it will be seen that the specific immune rabbit serum in various dilutions in combination with the bacterial polysaccharide gave positive fixation (tubes 1 and 2). Immune horse serum, on the other hand, did not exhibit this property (tubes 9, 10, 11). If immune horse serum was subsequently added to potentially active combinations of polysaccharide and immune

rabbit serum, the reaction was blocked in all instances except one in which the amount of immune horse serum was relatively small (tube 5).

It may be pointed out that the immune horse serum was added after the reaction between the polysaccharide and rabbit antibody had occurred. It is entirely conceivable that the antibodies of the immune

TABLE VII
The Reverse of the Inhibition Experiment

Tube	Type I pneumococcus capsular polysaccharide	Type I anti-pneumococcus horse serum		Type I anti-pneumococcus rabbit serum					Result (fixation)
	0.5 cc.	0.5 cc.		0.5 cc.					
1	1:100,000	1:5		—					—
2	"	1:25		—					—
3	"	1:5		1:2					+++
4	"	"		1:10					—
5	"	"		1:25					—
6	"	1:25		1:2					++++
7	"	"		1:10					++++
8	"	"		1:25					++++
9	"	—		1:2					++++
10	"	—		1:10					++++
11	"	—		1:25					++++
12	"	—	10 minutes 37°C.	—	10 minutes 37°C.	2 units guinea pig complement	30 minutes. 37°C.	1 cc. sensitized sheep cells	—
13	—	1:5		—					—
14	—	1:25		—					—
15	—	—		1:2					—
16	—	—		1:10					—
17	—	—		1:25					—

horse serum reacted in some way with the already formed rabbit aggregates. Thus it might be assumed that the core of the final aggregates was composed of rabbit antibody-polysaccharide combination with only the surface reoriented by the addition of the horse serum antibodies, in themselves not capable of bringing about fixation and thus transforming potentially active particles of immune precipitate into

inactive ones. If this assumption were true it should be possible to reverse the typical inhibition experiment, that is, to add the immune rabbit serum as the second reagent and thus bring about fixation. The results of an experiment of this type are shown in Table VII.

It will be noted that the capsular polysaccharide in combination with immune horse serum failed to give fixation (tubes 1 and 2) whereas the rabbit immune serum gave positive results (tubes 9, 10, 11). If, however, immune rabbit serum was added to combinations of the polysaccharide and immune horse serum, fixation was obtained in every instance in which the amount of rabbit serum was equal to or greater than the amount of horse serum.

These experiments demonstrate two facts. The first of these is that immune horse serum is not, as had been deduced by previous workers, of itself inhibitory. The important point is the order in which the two antibodies react with the polysaccharide. It is quite apparent that, in general, the serum last added confers its characteristic properties on the whole aggregate, that is, the second serum reagent masks the reaction expected with the first.

Secondly, there is an obvious quantitative factor. Thus in both experiments the reactive properties of the mixture were those of the second serum reagent in instances in which the concentration of the latter was as great or greater than that of the first serum reagent.

Although there is a possibility that the second antibody may unite with minute traces of uncombined polysaccharide, it seems probable that this reaction is of little quantitative consequence. Furthermore, the experiments in which immune horse serum was added to potentially fixing combinations of polysaccharide and immune rabbit serum yield conclusive evidence to support the assumption that the surface property of the preformed aggregates is reoriented by the second antibody.

The Type Specificity of the Inhibition Phenomenon

It would seem likely that if any actual "inhibitor" were involved it should be present in horse serum in general, and particularly in anti-pneumococcus horse sera of heterologous types. In order to test the possibilities of this assumption an experiment was arranged to include capsular polysaccharides of two types of pneumococci and their homologous immune horse and rabbit sera. The results of this experiment are shown in Table VIII.

From these results it will be noted that each type polysaccharide gave fixation with its homologous immune rabbit serum (tubes 1 and 6). Controls not reproduced in the protocol showed that these reactions were entirely type-specific. The addition of homologous type antipneumococcus horse sera to potentially fixing mixtures of the above reagents completely blocked the reactions (tubes 2, 3, 9, 10). On the other hand, heterologous type immune horse sera failed to have any effect (tubes 4, 5, 7, 8).

TABLE VIII
Specificity of the Inhibition Phenomenon

Specificity of the Inhibition Phenomenon									
Tube	Pneumococcus capsular polysaccharide 1:100,000	Antipneumococcus rabbit serum			Antipneumococcus horse serum				Result (fixation)
		Type	Dilution		Type	Dilution			
1	0.5 cc. I	0.5 cc. I	1:5	30 minutes 37°C.	0.5 cc. —	—	30 minutes 37°C.	2 units guinea pig complement	++++
2	"	"	"		I	1:5			
3	"	"	"		"	1:10			
4	"	"	"		II	1:5			
5	"	"	"		"	1:10			
6	II	II	"	30 minutes 37°C.	—	—	30 minutes 37°C.	1 cc. sensitized sheep cells	++++
7	"	"	"		I	1:5			++++
8	"	"	"		"	1:10			++++
9	"	"	"		II	1:5			++++
10	"	"	"		"	1:10			++++

Controls showed that none of the reagents were antigenic in amounts used, and that neither reagent was anticomplementary.

Controls showed that none of the reagents were anticomplementary in the amounts used, and that neither type of antipneumococcus horse serum gave fixation with the pneumococcus polysaccharides.

From these results it can be concluded that the inhibition is an entirely type-specific phenomenon. These results also suggest that any hypothetical inhibitor must be intimately associated with the specific antibody, for it is ineffective unless bound by the immune body to the reactive aggregate.

The Time Element in the Inhibition Phenomenon

In an hypothesis previously presented, it was suggested that the striking inhibition by immune horse serum might be related to the fact

that during the first incubation period immune aggregates are increasing in size, so that at the time the immune horse serum is added there is an appreciable diminution in surface, and hence the amount of immune horse serum need not be as large as the original amount of immune rabbit serum, in order to block the reaction. If this hypothesis were correct, the simultaneous admixture of the reagents should give results quantitatively different from those obtained by following the usual procedure.

Table IX shows the results of an experiment in which the two sera were mixed before the addition of the polysaccharide, in comparison with the results of a similar experiment in which the immune horse serum was added 30 minutes after the admixture of polysaccharide and immune rabbit serum.

TABLE IX
Simultaneous Addition of the Two Sera

Immune rabbit serum	Immune horse serum			
	1:10		1:25	
	Added immediately	Added after 30 min.	Added immediately	Added after 30 min.
1:5	++++	+++	++++	++++
1:10	++++	—	++++	+++
1:25	++	—	++++	—
1:50	—	—	++++	—

Acetyl polysaccharide 1:100,000 in all instances.

The results presented of this experiment demonstrate that, with the simultaneous exposure of the polysaccharide to both sera, the presence of immune horse serum did not block fixation except in those instances in which it was present in excessive proportions. On the other hand, in the typical inhibition experiment in which the immune horse serum was added after an interval of 30 minutes, inhibition was obtained when the amount of horse serum was equal to or greater than that of the immune rabbit serum.

Perhaps the most striking experiment in the series presented by Zinsser and Parker dealt with the observation that the interfering effect of the horse serum could be eliminated by absorption with pneumococci previously sensitized with immune rabbit serum. The authors state that the reported experiment was only suggestive and that

repeated experiments on this point had in general been inconclusive. A primary objection to this conception is, of course, the already established fact of the marked specificity of the reaction. If an inhibitor exists as such it is undoubtedly an intimate part of the antibody molecule and thus its selective removal should be difficult.

Repeated experiments directed toward the selective absorption of the inhibitor substance have been carried out. These have been uniformly unsuccessful. It is conceivable that absorption of the immune horse serum with pneumococci previously sensitized with immune rabbit serum might under proper conditions result in the partial replacement of the rabbit antibody by the horse antibody with the consequent release of the former. Should the rabbit antibody be released in an amount equivalent to or greater than that of the remaining horse antibody a fixing element might be detected in the supernatant and thus give the impression that the inhibitor had been absorbed from the horse serum. However, on the basis of our present knowledge regarding equilibria in these systems it is doubtful if the concentration of the free rabbit antibody could reach a point greater than that of the free horse antibody. Furthermore, it would seem unlikely that the reaction could continue to a point of complete replacement. Even if the reaction should reach an equivalent point and thus, in theory, render the supernatant fluid potentially active from the point of view of fixation it is extremely doubtful if it could be detected by this technique. In our experience the minimum concentration of the released antiserum would be of necessity greater than the equivalent of a 1:200 dilution of original immune rabbit serum, since with higher dilutions it is extremely difficult to demonstrate complement fixation.

For the purpose of detecting smaller amounts of the rabbit antibody, fowls were immunized with antipneumococcus rabbit serum. The sera of these immunized fowls reacted with rabbit serum in dilutions as great as 1:100,000. With this reagent a large number of experiments have been carried out to demonstrate the release of rabbit antibody both from sensitized pneumococci and from immune precipitates after these had been treated with homologous immune horse serum. These experiments are not reproduced in detail because of their inconclusive character. In about one-third of the total number no rabbit protein was detected in the supernatant fluid. In another third, including all of the experiments with immune precipitates, the controls, untreated with immune horse serum, showed traces of free rabbit protein in the supernatant fluid. In a very few instances minute amounts of rabbit protein appeared to be released after treatment of agglutinates with immune horse serum. These quantities were too small to possess any actual significance.

DISCUSSION

The experiments reported in this paper support the view that the failure to obtain complement fixation with combinations of pneumo-

coccus capsular polysaccharide and specific immune horse serum is not due to some heterologous inhibitor present in immune horse serum but is to be referred rather to some property of the horse antibody itself or some property of the immune aggregate resulting from the union of this antibody and the polysaccharide. It has been somewhat generally assumed that the resultant of an immunological reaction of antigen and antibody has the property of fixing complement. There are, however, instances other than the one cited in which this is not true. Certain information on this subject has been assembled in Table X. Pneumococcus proteins, for example, give fixation in both horse and rabbit immune sera (7). The M or specific protein frac-

TABLE X
Capacities of Certain Bacterial Derivatives to Fix Complement in Combination with Specific Precipitating Sera

Bacterial derivative	Complement fixation with	
	Immune rabbit serum	Immune horse serum
Pneumococcus protein (7).....	+	+
M (specific protein) fraction of <i>Streptococcus hemolyticus</i> (8).....	+	—
Pneumococcus capsular polysaccharide (1).....	+	—
<i>Hemophilus influenzae</i> capsular polysaccharide (2).....	+	—
C or somatic carbohydrate of Pneumococcus (9).....	—	—
C or somatic carbohydrate of hemolytic streptococcus (8).....	—	—

tion of *Streptococcus hemolyticus* gives fixation with immune rabbit serum (8). The capsular polysaccharides of Pneumococcus and of *Hemophilus influenzae* give fixation only with immune rabbit sera of homologous type (2). The C or somatic carbohydrate of the Pneumococcus fails to give complement fixation with either antipneumococcus horse or rabbit serum (9). A similar failure is obtained with the C or somatic carbohydrate of *Streptococcus hemolyticus* and its homologous immune rabbit serum (8). It must be emphasized that precipitation occurred in all of these instances. A properly controlled positive complement fixation result, therefore, indicates a specific reaction between antigen and antibody, but a failure in complement fixation does not necessarily imply the lack of specific combination.

It would appear from the reports of many workers, particularly Heidelberger and Kendall (10), and Boyd and Hooker (11), that each molecule of pneumococcus capsular polysaccharide is capable of uniting with varying numbers of specific antibody molecules. Similarly it does not seem improbable that a single polysaccharide molecule may unite at one and the same time with antibody molecules of two immune sera derived from two animal species. Whether or not this assumption is true, the experiments have shown that the results of the complement fixation test depend upon which of the two immune sera is present in greater amount, and that, within certain quantitative limits, one serum does not inhibit the action of the other providing both are introduced at the same time. If, on the other hand, one of these sera is added after a definite interval to a system containing the other serum and the antigen, one encounters what has been spoken of as an inhibition phenomenon. It has been shown that under certain quantitative conditions the second serum component predicates the result, and that the reaction of inhibition is type-specific and therefore directly concerned with the capacity of the secondarily added antibody to unite with exposed but unsaturated linkages of the polysaccharide. The results suggest that the effect of the second immune serum is in reorientation of the surface composition of the already formed immune aggregates. This is based on the fact that the amount of the second serum need not be as great as that of the first.

These deductions with reference to the surface phenomena are also supported by the findings as to the amount of complement fixed by combinations of immune rabbit serum and specific polysaccharides. Thus in several types of experiments it was shown that greater amounts of complement were fixed the greater the surface exposure of the immune aggregates. Surface exposure in non-static systems of this order is obviously an expression of the sums of surfaces over progressive time intervals minutely spaced, that is, a true surface-time function, but in its simplest conception it is resolved into total surface.

Moreover, these results support the view that the fixation of complement is a phenomenon of selective adsorption. That one type of aggregate absorbs complement while another fails to do so is curious, but far from unique. A close parallelism is to be found in the fact that horse antibody-polysaccharide aggregates adsorb cephalin, while aggregates containing rabbit antibody selectively adsorb lecithin (12).

It has been clearly shown that complement fixation does not occur in the absence of particulation. When the union of antigen and antibody results in a perfectly soluble combination, complement is not fixed. Since particulation is in itself a secondary phenomenon, complement fixation must be regarded as at least tertiary.

SUMMARY

1. Complement is not fixed by immune aggregates resulting from the interaction of pneumococcus capsular polysaccharide and type-specific immune horse serum, although under proper conditions the substitution of immune rabbit serum gives positive results.
2. The negative results with immune horse serum are due to some poorly understood property of the specific antibodies rather than to some heterologous inhibitor present in the serum.
3. It has been shown that with immune rabbit serum-polysaccharide combinations, complement fixation is an adsorptive phenomenon conditioned upon the surface exposure of the immune aggregates.
4. A close parallelism to the selective adsorption of phosphatides by these immune aggregates has been pointed out.
5. In those instances in which complement is fixed this phenomenon must be regarded as tertiary and conditioned by (a) union of antigen and antibody, and (b) particulation.
6. The general significance of complement fixation as applied to bacterial polysaccharides has been discussed.

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ACTIVE IMMUNIZATION OF GUINEA PIGS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

III. QUANTITATIVE STUDIES OF SERUM ANTIVIRAL BODIES IN ANIMALS IMMUNIZED WITH ACTIVE AND INACTIVE VIRUS

BY HERALD R. COX, Sc.D., AND PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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In the first paper of this series (1) a basis was constructed for determining the minimal amount of untreated active virus of equine encephalomyelitis needed for the successful production of resistance in guinea pigs to 10^3 or 10^4 intracerebral lethal doses of the virus. In the second article (2) we showed that formolized material induced, within certain limits, as high a degree of resistance as did untreated active virus, and this refractory state was shown not to be due to the action of "living" virus as such in the formolized immunizing preparation.

The results of a study on humoral antibodies in guinea pigs treated with known quantities of active virus and of virus inactivated by formalin (2) will be described in the present communication. It was necessary first to determine the optimal conditions by which the presence of serum antiviral bodies could be brought to light; and having found the best means for their demonstration, to note any difference in content of antibody occurring in the serum of animals immunized with active and with inactive virus. The findings served to explain certain phases of the mechanism of the resistance developed by guinea pigs after injections of formolized inactive virus.

In early work on equine encephalomyelitis virus, Meyer, Haring, and Howitt (3) reported that sera of spontaneously recovered or resistant horses failed to neutralize homologous virus, while those of recovered rabbits, guinea pigs, and monkeys neutralized only irregularly. It was later shown that antiviral bodies were demonstrable in convalescent animals only when serum was added to low multiples of M.L.D. of virus (4, 5, 6). Howitt (4, 7) recorded more constant pres-

ence of serum antibodies in recovered animals, especially horses, after hyperimmunization by means of reinjection with active virus. In guinea pigs, moreover, tissue immunity conferred by vaccination was found to last longer than humoral: resistance to an intracerebral test was therefore present without demonstrable neutralizing bodies in the blood, but the converse could not be proved. Hurst (8) found antiviral substance in the blood of surviving monkeys as early as the 4th day after peripheral inoculation of virus. Neutralization of 1 to 10 M.I.D. of virus as determined by the mouse intracerebral test could "almost invariably" be effected—in some instances even 100 to 1,000 M.I.D. Monkeys that withstood a later intracerebral or intramuscular injection of large amounts of virus did not reveal an appreciable increase of the neutralizing titer of their sera.

Viewing the recorded experiments on the production of serum antiviral bodies in animals recovered from infection or immunized by virus inoculations, one observes that the antibodies occur irregularly or in low concentration.

Materials and Methods

Virus.—The Eastern strain of equine encephalomyelitis virus was employed as fresh or glycerolated guinea pig or mouse brain tissue. Its activity was measured by the results of intracerebral injection of mice (1).

Animal Inoculation.—Guinea pigs were immunized with active or formolized virus, as already described (1, 2). In most instances mouse brain passage virus was used as immunizing preparation and in all cases wherein guinea pigs were tested for induced immunity, the homologous passage virus was injected intracerebrally.¹

Serum.—Pooled immune serum was employed, being derived from groups of animals, all of which were treated alike with either active or inactive virus. Sera were collected, as a rule, from 4 to 8 days before tests were made. Hyperimmune guinea pig serum was obtained from animals surviving intracerebral inoculation of virus and which, in addition, had resisted three later subcutaneous injections of 3×10^8 to 6×10^8 mouse intracerebral infective units (m.i.u.) of virus (1, 2), given at weekly intervals. These animals were bled for collection of serum 8 to 10 days after the last subcutaneous dose.

Neutralization Tests.—A preliminary test was always made to determine the infectivity of a stock virus suspension which consisted of infective mouse brain ground in hormone broth pH 7.4 and spun in an angle centrifuge at 3,000 R.P.M. for 20 minutes. This suspension, when stored at 5°C., was found to retain its original infectivity for at least 14 days. In conducting the neutralization test,

¹ Operative procedures on animals were performed with the aid of ether anesthesia.

TABLE I

Comparison of the Antiviral Content of Sera of Guinea Pigs Treated with Active and with Formolized Virus. Serum-Virus Mixtures Held at 20 to 22°C. for 15 Minutes before Injection

Experiment No.	No. of sera pooled	Immunized with	No. of weekly injections	M.i.u. of virus in each dose	Time between 1st inoculation and serum collection	Survivors in treated group after intracerebral test of >1,000 M.L.D. given 24 to 36 days after inoculation	Mice survivors after intracerebral injection of serum-virus mixtures					
							Virus dilutions (absolute)					
							10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹
1	4	A V	1	3 × 10 ²	days	29	0/3	0/3	0/3	0/3	0/3	3/3
	4	"	3	3 × 10 ²	22	0/4	0/3	0/3	0/3	0/3	2/3	3/3
	5	"	1	3 × 10 ³	29	3/5	0/3	0/3	0/3	0/3	2/3	3/3
	5	"	3	3 × 10 ³	22	5/5	0/3	0/3	0/3	2/3	2/3	3/3
	5	"	1	3 × 10 ⁴	28	2/5	0/3	0/3	0/3	0/3	2/3	2/3
	5	"	1	3 × 10 ⁵	30	5/5	0/3	0/3	0/3	3/3	2/3	3/3
	6	"	2	3 × 10 ⁵	28	6/6	0/3	0/3	0/3	1/3	1/3	3/3
	5	"	1	3 × 10 ⁷	30	5/5	0/3	0/3	0/3	2/3	3/3	3/3
	3	F V II	2	N T	21	3/3	0/3	0/3	0/3	0/3	1/3	3/3
	4	F V IX	2	"	28	8/8	0/3	0/3	0/3	0/3	2/3	2/3
	4	F V XIV	1	3 × 10 ^{7*}	31	s c test 4/4	0/3	0/3	0/3	0/3	3/3	3/3
	4	" "	2	"	32	5/5	0/3	0/3	0/3	1/3	3/3	3/3
	4	F V XV	2	"	19	5/5	0/3	0/3	0/3	1/3	1/3	3/3
2	4	" "	2	1.5 × 10 ⁸	19	5/5	0/3	0/3	0/3	1/3	3/3	2/3
	4	(conc. 5-fold)										
	4	Normal guinea pig sera				0/4	0/3	0/3	0/3	0/3	0/3	3/3
	5	A V	3	3 × 10 ³	22	5/5	—	0/3	0/3	1/3	1/3	3/3
	5	"	1	3 × 10 ⁷	30	5/5	—	0/3	0/3	2/3	3/3	3/3
	4	F V XIV	2	3 × 10 ⁷	32	5/5	—	0/3	0/3	0/3	3/3	3/3
	4	F V XV	2	1.5 × 10 ⁸	19	5/5	—	0/3	0/3	2/3	2/3	3/3
		(conc. 5-fold)										
	10	Hyperimmune guinea pig sera				10/10	—	1/3	3/3	3/3	3/3	3/3
	4	Normal guinea pig sera				0/4	—	0/3	0/3	0/3	0/3	3/3

In this and the succeeding table the numerator indicates the number of animals surviving; the denominator, the number of animals injected.

A V = active virus; F V = formolized vaccine, the number following representing the particular vaccine used; i c = intracerebral; s c = subcutaneous; N T = not titrated for virus content prior to formolization.

* Titration prior to formolization revealed 3 × 10⁷ m.i.u. per cc.

the stock virus was diluted decimally in broth to 1:500,000,000 and to each dilution was added an equal volume of undiluted serum. The resulting serum-virus mixtures were tested soon after mixing (kept up to 20 minutes at room temperature) or after retention for 2 or 3 hours at 37°C. Each of three mice was injected intracerebrally with 0.03 cc. of a serum-virus preparation.

EXPERIMENTAL

In Table I is shown a comparison of the antiviral body content of the sera of guinea pigs treated with active virus and with formalized vaccine. The different serum-virus mixtures were tested at the same

TABLE II
Comparative Effect of Incubation in Revealing Antiviral Bodies in Serum-Virus Mixtures

Sera	Serum-virus mixtures injected											
	up to 20 minutes after mixing						after 2½ hours at 37°C.					
	Virus dilutions						Virus dilutions					
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Normal guinea pig	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3
Guinea pigs injected twice at 7 days' interval with F V XV. Bled 19 days later	0/3	0/3	0/3	0/3	2/3	3/3	0/3	0/3	0/3	2/3	3/3	3/3
Guinea pigs injected once with 3 × 10 ⁵ m.i.u. A V. Bled 30 days later	0/3	0/3	0/3	0/3	3/3	3/3	0/3	0/3	0/3	2/3	3/3	3/3
Hyperimmune guinea pig	0/3	0/3	1/3	2/3	3/3	3/3	0/3	2/3	3/3	3/3	3/3	3/3

Abbreviations as in Table I.

time and in each case were held at room temperature not longer than 20 minutes before being injected intracerebrally in mice. It is apparent that under these conditions only the hyperimmune guinea pig serum revealed an appreciable quantity of virus antibodies, *i.e.*, neutralizing 100 to 1,000 M.I.D. The sera derived from guinea pigs which resisted the intracerebral test for induced immunity exhibited partial or complete protection against only 1 to 10 M.I.D. of virus. Guinea pigs which had received an amount of virus inadequate to

bring about immunity against the intracerebral test for resistance possessed no significant humoral protective properties. Finally, no distinct difference could be seen in antibody content of serum collected from guinea pigs treated with active or inactive virus.

Table II summarizes the experiments on the relation of incubation to titration of humoral antiviral bodies. Incubation clearly enhanced the protective substance in equine encephalomyelitis antisera. Here a tenfold increase was demonstrable in all of the immune sera tested. Hence to reveal small amounts of antiviral bodies it is best to incubate serum-virus mixtures before animal inoculation.

SUMMARY AND DISCUSSION

An analysis of the preceding experiments discloses that antiviral bodies are demonstrable not at all or in small amounts in the sera of guinea pigs injected with a quantity of active virus not sufficient to induce immunity against the described intracerebral test for induced resistance. However, neutralizing bodies are found in immune animals, although in low concentration, and are regularly manifested when serum is added to low multiples of infective doses of virus under optimal conditions of time and temperature. Hyperimmune serum, on the other hand, reveals a distinct increase in the amount of antiviral bodies present.

Irrespective of the mode of procedure for revealing neutralizing bodies, there does not appear to be any notable difference in the content of such bodies in the serum of animals immunized with active virus or with formolized vaccine in which active virus could not be demonstrated. In other words, the antigenic complexes in active as well as in inactive virus produce similar degrees of antibody reaction. The formolization of virus tissue suspensions, therefore, can be considered as a process whereby the virus is inactivated but the antigenicity of the suspensions is preserved, as is also shown in the preceding paper of this series in tests on tissue immunity. In that article is described the remarkably high degree of tissue immunity which results from injections of inactive virus; now we demonstrate that this resistance is associated with a minimal degree of serum antibody.

Finally, the question may well be asked, if practically no antiviral bodies are demonstrable immediately or soon after mixing immune

serum and virus, and are recognizable in a tenfold increase when functions of time and temperature are brought into play, whether the bodies are "neutralizing" or the phenomenon is due merely to aggregation of virus particles by the serum. From the recent work on the same virus and immune serum (9) by Merrill, there appears to be warrant for the belief in aggregation of virus particles which in turn diminishes the virus activity to the indicated degree.

CONCLUSIONS

Guinea pigs injected with amounts of active equine encephalomyelitis virus inadequate to induce protection against an intracerebral test of 1,000 or more M.L.D. of virus show no significant humoral antiviral bodies. The latter are, however, regularly present in immune animals and are best demonstrated by adding serum to low multiples of infective doses of virus under optimal conditions of time and temperature ($2\frac{1}{2}$ hours at $37^{\circ}\text{C}.$). Guinea pigs immunized either with active or with inactive (formolized) virus reveal no distinctive differences in the antiviral body content of their sera.

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ACTIVE IMMUNIZATION OF GUINEA PIGS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

IV. EFFECT OF IMMUNE SERUM ON ANTIGENICITY OF ACTIVE AND INACTIVE VIRUS

By HERALD R. COX, Sc.D., AND PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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In one of the preceding papers of this series (1) it was shown that formolization of tissue suspensions containing the active virus of equine encephalomyelitis inactivates the virus. This is reflected by the complete loss of infectivity of even large amounts of material after inoculation by various routes in guinea pigs and mice (1, 2). Yet formolized vaccines induce in these animals a high grade of resistance, as evidenced by development of immunity to as much as 1,000 to 10,000 M.L.D. of virus given intracerebrally. The protection afforded compares favorably, within certain limits, with that evoked by untreated active virus—indeed with the same suspensions of active virus employed in the preparation of formolized vaccines. In another article (3) reference was made to the fact that the antigenic complex existing in active as well as in inactive virus produces practically the same amount of humoral antiviral bodies. The formolization of tissue suspensions of equine encephalomyelitis virus can therefore be regarded as a means of inactivating the virus and at the same time of preserving the antigenicity of the suspension.

In this communication a study is presented on the effects of immune serum on the antigenic capacity of active and of formolized virus.

Methods and Materials

The methods and materials as employed in these experiments, including the Eastern strain of equine encephalomyelitis virus, have already been described in the preceding paper (3). Since guinea pig hyperimmune serum was found to be more potent than ordinary immune serum, the former (Tables I and II, Paper III (3)) was used. This serum, under optimal conditions of time and temperature

(2½ hours at 37°C.), neutralized 1,000 to 10,000 M.L.D. of virus, as manifested by the results of the mouse intracerebral test (3).

The mode of procedure in an experiment was as follows: 1 cc. of hyperimmune serum was injected into subcutaneous tissue of the abdomen of guinea pigs and 1 hour later, 1 cc. of active or formolized virus was inoculated under the skin of the opposite side. The animals received two such courses of treatment at 7 days' interval and were tested for induced immunity by intracerebral inoculation¹ of > 1,000 < 10,000 M.L.D. of virus given 16 days after the last immunizing dose. It will be observed from the protocols to be presented that the amount of serum alone, as given, did not suffice to confer passive immunity.

Immune Serum Followed by Active Virus

In a preliminary experiment, one series of guinea pigs was given active virus alone and another undiluted immune serum followed within an hour by the same virus. The outcome yielded results which prompted us to conduct another experiment with different dilutions of immune serum and with both active and inactive virus.

The results of the preliminary test are summarized in Table I. When active virus alone was used, of guinea pigs receiving two injections of 3×10^5 or more mouse infective units of virus (m.i.u.) (1, 4), seven of seventeen succumbed to virus infection during the period of immunization, the incidence of death being proportional to the amount of virus introduced. Of the survivors, however, all proved to be resistant to an intracerebral test of > 1,000 < 10,000 M.L.D. of virus. On the other hand, those given 3×10^4 or less m.i.u. of virus survived the treatment but only one of eight was found immune to the test for induced resistance. The findings are in agreement with our prior observations (1, 4).

When undiluted hyperimmune serum was injected in advance of the virus, all guinea pigs survived the two courses of inoculation, but none of them proved to be resistant to the described intracerebral test. In other words, in each instance and with concentrations of virus ranging from 3×10^3 to 3×10^8 m.i.u., the relatively small amount of immune serum prevented the antigenic action of the suspensions of active virus.

This blocking effect of undiluted immune serum on the antigenic stimulus of active virus might be considered merely as the result of

¹ All such operations were carried out with the aid of ether anesthesia.

preventive action of the serum on multiplication of the virus after its introduction in the body, thus producing less antigenic substance and in turn no resistance on the part of the host. That this is not the mode of action can be deduced from the next series of experiments. These were planned to determine the results yielded by smaller

TABLE I

Guinea Pigs Receiving Hyperimmune Guinea Pig Serum and Active Virus or Active Virus Alone

Quantity of undiluted serum in each dose	M.i.u. of virus in each dose	Animals showing fever of 104°F. or above	Animals surviving virus infection during immunization period	Immunity test
				Survivors of an intracerebral test of >1,000 <10,000 M.I.D. given 16 days later
1 cc.	3×10^8	3/4	4/4	0/4
None	" "	4/4	1/4	1/1
1 cc.	3×10^7	2/4	4/4	0/4
None	" "	2/4	3/4	3/3
1 cc.	3×10^6	0/4	4/4	0/4
None	" "	3/4	2/4	2/2
1 cc.	3×10^5	2/4	4/4	0/4
None	" "	1/5	4/5	4/4
1 cc.	3×10^4	1/4	4/4	0/4
None	" "	1/4	4/4	1/4
1 cc.	3×10^3	1/4	4/4	0/4
None	" "	0/4	4/4	0/4
1 cc.	None	0/4	4/4	0/4

Animals were given two courses of serum and virus or virus alone at 7 days' interval. In instances in which serum was used, it was followed an hour later by a subcutaneous injection of active virus on the opposite side. Weight of animals = 275 to 325 gm. Denominator represents the number of animals in each test.

amounts of serum and to disclose any blocking effect of both diluted and undiluted immune serum exerted against virus inactivated by formalin. For in the latter material no active virus could be demonstrated (1)—hence no probability of its multiplication *in vivo*—and still a sufficient amount of antigenic substance was present to induce a high degree of immunity (1).

Immune Serum Followed by Active and Inactive Virus

In the following experiment devised to reveal any comparable preventive effect *in vivo* of immune serum on the antigenic capacity of active and of inactive virus, the antigens employed consisted of two materials. The first was active virus as was present in the brain of mice that succumbed to experimental encephalomyelitis. This was employed in doses of 3×10^7 m.i.u., a potent immunizing quantity, and in doses of 3×10^5 m.i.u., the minimal quantity required to immunize guinea pigs against an intracerebral test of 1,000 M.L.D. of virus. The second consisted of formolized vaccine. The last preparation which was shown to be free of demonstrable active virus (1) was made from the same virus tissue suspension employed in the first material just mentioned, and hence contained 3×10^7 m.i.u. before formolization. The data of this experiment are shown in Table II and are summarized as follows:

Untreated, Active Virus (3×10^7 M.i.u.) as Antigen.—With a dose of 3×10^7 m.i.u. of virus given 1 hour after the same undiluted serum as was described in Table I, all animals survived the courses of treatment but none became immune. With 1:4 dilution of serum, again all animals survived; they were, however, proved to be resistant to a later intracerebral test. Finally, with 1:8 or higher dilutions of serum, ten of thirty-one animals succumbed to virus infection during the period of immunization, and all survivors withstood the intracerebral test of $>1,000 <10,000$ M.L.D. given 20 days after the last immunizing dose.

Untreated, Active Virus in Minimal Immunizing Quantity (3×10^5 M.i.u.) as Antigen.—When 3×10^5 m.i.u. of virus was used, preceded by an injection either undiluted or 1:4 dilution of immune serum, all animals so inoculated lived through the treatment but only one of eight was found to withstand the intracerebral test for induced immunity. With 1:8 and 1:16 dilutions of serum, all animals survived the subcutaneous injections; three of eight failed, however, to resist the immunity test. With 1:32 or higher dilutions of serum, four of twenty-four guinea pigs died of virus infection during the courses of injections but all survivors except one were successfully immunized to the test dose.

Virus Inactivated by Formalin.—Of eight guinea pigs that received formolized vaccine preceded by the administration of either undiluted or 1:4 dilutions of serum, none succumbed during the period of this treatment and none was found immune to the later intracerebral test. With 1:8 and 1:16 dilutions of serum, all animals withstood the injections and only three of eight the test for immunity. The twenty-four guinea pigs given 1:32 or higher dilutions of serum survived the inoculations, and all but two, the later intracerebral test of $>1,000 <10,000$ M.L.D. of virus.

TABLE II

Vaccination of Guinea Pigs with Hyperimmune Guinea Pig Serum and Active or Formolized Virus

Dilution of serum	M.i.u. of virus in each dose		Animals surviving virus infection during immunization period	Immunity test
	Active virus	Formolized vaccine		Animals surviving an intracerebral test of >1,000 <10,000 M.L.D. given 20 days later
Undiluted	3×10^7	—	4/4	0/4
	3×10^5	—	"	"
	—	3×10^7	"	"
1:4	3×10^7	—	"	4/4
	3×10^5	—	"	1/4
	—	3×10^7	"	0/4
1:8	3×10^7	—	2/3*	2/2
	3×10^5	—	4/4	2/4
	—	3×10^7	"	"
1:16	3×10^7	—	"	4/4
	3×10^5	—	"	3/4
	—	3×10^7	"	1/4
1:32	3×10^7	—	2/4	2/2
	3×10^5	—	3/4	2/3
	—	3×10^7	4/4	3/4
1:64	3×10^7	—	3/4	3/3
	3×10^5	—	"	"
	—	3×10^7	4/4	4/4
1:128	3×10^7	—	1/4	1/1
	3×10^5	—	4/4	4/4
	—	3×10^7	"	3/4
1:256	3×10^7	—	3/4	3/3
	3×10^5	—	4/4	4/4
	—	3×10^7	"	"
1:512	3×10^7	—	2/4	2/2
	3×10^5	—	3/4	3/3
	—	3×10^7	4/4	4/4
1:1,024	3×10^7	—	"	"
	3×10^5	—	3/4	3/3
	—	3×10^7	4/4	4/4

Animals received two courses of serum and virus at 7 days' interval. Serum injection followed an hour later by an injection of either active or formolized virus on the opposite side. Weight of animals = 275 to 325 gm.

*One of the animals in this group died of an intercurrent, streptococcal infection.

From these experiments it is evident that hyperimmune serum in sufficient quantity injected shortly before the antigen can prevent or block the immunizing action not only of untreated active virus but also of formolized inactive virus. Indeed, the preventive action of 1 cc. of serum is effectively exerted against about 30 million (Table II) to 300 million (Table I) infective units of virus; less serum is needed, however, to block the antigenic capacity of the smaller amount of active agent (300,000 infective units) than of the larger. At this point attention is directed to the fact that with the same serum dilutions, similar results were obtained with formolized vaccine to those with 3×10^5 m.i.u. of active virus—the minimal effective immunizing dose (Table II).

Relation to Immunization with Serum-Virus Combinations.—These experiments, taken together with others carried out by Howitt (5), whose results have been confirmed by our own observations, also have a bearing on the immunization of guinea pigs by means of combinations of immune serum and active virus. It should be stressed that both materials should be introduced into the animal in certain definite proportions if the desired immunity without death from virus infection during the period of immunization is to be attained. Thus with the particular hyperimmune serum studied (Table II) and with 3×10^7 m.i.u. of virus, the optimal conditions for inducing immunity were achieved by employing 1:4 dilution of serum; with 3×10^5 m.i.u., 1:16 to 1:64 dilutions. Furthermore, the proper dilution of each individual immune serum sample should be determined by test with each individual quantity of active virus employed.

Equine encephalomyelitis virus differs in this respect from certain other virus agents, such as yellow fever, in which "when the optimal amount of immune serum has been ascertained, a wide range of virus concentration can be used with success" (6) for immunization. It is clear, nevertheless, that formolized vaccines containing inactivated equine encephalomyelitis virus could replace injections of combined serum and virus for the immunization of guinea pigs with elimination of the necessary, laborious, serum titrations.

It has been assumed that generally antiserum in neutralized and overneutralized suspensions of virus employed for artificial immunization of animals, acts to prevent the development of immunity by

inhibiting the multiplication of virus and thus the production of more antigen in the body. The results of the foregoing experiments show that in so far as the encephalomyelitis virus is concerned, the serum blocks the development of immunity in the instance in which suspensions containing inactivated, nonmultiplying virus are used as immunizing preparations.

SUMMARY AND DISCUSSION

A study was undertaken on the effect *in vivo*, in the guinea pig, of equine encephalomyelitis virus antiserum upon the antigenic response to active, as compared with that to formolized, inactive virus. It was found that when animals were given subcutaneously a proper amount of hyperimmune serum 1 hour before inoculation, in the subcutis, of either active or of inactive virus, no immunity was induced against an intracerebral test of more than 1,000 and less than 10,000 M.L.D. of virus. This preventive power of the serum was lost by its dilution, the loss being proportional to the dilution, and, on the other hand, more serum was needed to obtain the blocking effect as the quantity of virus was increased. When an insufficient amount of serum was introduced into the animals along with the same quantities of active virus or formolized vaccine, a certain number of those receiving the untreated virus succumbed to virus infection in the course of the inoculations, but the survivors were rendered resistant to the intracerebral test; all the guinea pigs treated with higher dilutions of serum and with formolized material were brought safely to an immune state.

The point to be stressed then is that antigenic stimuli present in untreated active virus and in formolized virus tissue suspensions in which no active virus is demonstrable by drastic tests (1) and which are wholly noninfective in animals (1), are completely inhibited from acting by the use of proper amounts of immune serum.

The mechanism underlying this preventive power of adequate amounts of serum may be explained on the basis of facts deduced in preceding papers of this series (1, 3) and in the present article. We have shown that 3×10^7 m.i.u. of active virus contains a sufficient amount of antigen to induce immunity without the necessity of its multiplication in the animal body. This has been fully established by the similar degree of resistance brought about by 3×10^7 m.i.u. of

virus formolized to a degree in which no active virus could be revealed (1). The assumption that the blocking effect of serum in the quantity employed prevents multiplication of the virus which is reflected in the production of inadequate amounts of antigen, is therefore untenable, since this effect was obtained when a sufficient amount of antigen was present in "living" as well as in "killed" virus. On the other hand, with insufficient amounts of immune serum (to be noted in higher dilutions shown in Table II), only the active virus could multiply—the formolized vaccine was not affected in respect to its antigenicity by these quantities of serum—and so produce more antigenic substance. This substance, in turn, brought about greater resistance in the host.

The precise action of proper amounts of serum in preventing development of immunity by both active and inactive virus is not definitely known. However, two hypotheses are offered for consideration: the first implies that the action of the serum is direct, that is, by entering into combination with the antigens to bar antigenic capacity; the second ascribes to the serum an indirect action, on the cells of the body, in such a way as to make them unable to react to the antigenic stimuli present in the inoculated materials.

The identity of these antigenic stimuli in virus suspensions containing the active, infective agent or this agent inactivated by formalin is at the present time undetermined. If virus were obtainable in pure state, free from extraneous material, the answer to this question might be readily given, but it is quite a different matter when the substance called virus is a mixture of the infective agent, of inflammatory tissue products, of tissue, etc. We have, however, shown that induced immunity is not due to the presence of "living" virus, but whether the antigenic action originates from "killed" virus or from another constituent of the suspension is not clear. On the other hand, Sabin (7) suggests the possibility that the virus may not be the direct antigenic stimulus but that some substance on which it acts and which becomes liberated from infected cells may be the factor responsible. While this subject awaits the results of further study, we believe that formalin inactivates the infective agent in virus suspensions and preserves the antigenic component therein, whatever its nature may be.

It would be of interest if this phenomenon of prevention of antigeni-

capacity by proper amounts of immune serum might apply to such materials which by their very nature do not multiply in the body of the host, *e.g.*, toxin and antitoxin. Theobald Smith (8) and later Park (9) demonstrated that in mixtures of diphtheria toxin-antitoxin, when smaller amounts of immune serum (antitoxin) are used, the toxicity of the mixture is retained and immunity results; if the serum is increased, toxicity is reduced and immunity occurs irregularly, and if more serum is added, no toxicity nor immunity results. This is supported by the experiments of Hartley (10) on washed precipitates from underneutralized, neutral, and overneutralized mixtures of antitoxin and toxin: those derived from underneutralized material are toxic and powerfully antigenic; those from neutralized, atoxic and of good antigenic action, and from overneutralized, atoxic and of low antigenicity. Hartley states, moreover, that the precipitate reactions of toxicity and antigenicity bear a close relationship to the nature of the mixture from which they are produced.

There is, therefore, a connection between the preventive reactions of the serum on the two forms of virus and of antitoxin on toxin in respect to toxicity and antigenicity. Furthermore, the toxin is rendered atoxic with retention of immunizing capacity by formalin: the production of toxoid or anatoxin (Glenny and Hopkins (11), Ramon (12))—again a condition related to the effects following formolization of the virus. It has, however, been stated that “in an immunizing mixture prepared with modified [formolized, but partially detoxified] toxin the antitoxin present does not within wide limits affect the antigenic power” (Glenny, Hopkins, and Pope (13)). It is not known whether a preliminary injection of antitoxic serum could have prevented the antigenic power of fully detoxified toxin, that is, after the passive immunity induced by the serum disappears. If a preventive action of antitoxic serum could be shown under these circumstances, a remarkable correlation of the reactions of proper amounts of antitoxin to toxoid and of proper amounts of immune serum on the virus would be evident.

Finally, the inhibition of antigenic power of both active and inactive virus by immune serum has been demonstrated to apply to the virus of equine encephalomyelitis in guinea pigs and no generalizations of the application of the phenomenon to other viruses are intended.

CONCLUSION

In active equine encephalomyelitis virus and in the virus inactivated by formalin, there is a sufficient amount of antigen, without necessity of multiplication in the body, to produce immunity in guinea pigs against $>1,000 <10,000$ intracerebral lethal doses of virus. The antigenic capacity of both materials can be blocked to the same extent by the action of an appropriate amount of hyperimmune serum. The bearing of these findings on the mechanism of immunity induced by virus inactivated by formalin is discussed.

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STUDIES ON THE SUPRARENAL CORTEX

V. THE INFLUENCE OF THE CORTICAL HORMONE UPON THE EXCRETION OF WATER AND ELECTROLYTES IN THE SUPRARENALECTOMIZED DOG*

By GEORGE A. HARROP, M.D., WILLIAM M. NICHOLSON, M.D., AND MARGARET STRAUSS

(From the Chemical Division of the Medical Clinic, Johns Hopkins University and Hospital, Baltimore)

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In previous papers (1) we described some of the physiological changes in the suprarenalectomized dog that follow the cessation of injections of the cortical hormone. It was shown, as had been reported by various observers who studied the results of bilateral suprarenalectomy (2), that a shock-like condition develops which is associated with the features of dehydration, and terminally with circulatory collapse. We subsequently reported that almost at once after withdrawal of injections of cortical extract from such animals there was a marked rise in the excretion of urinary sodium (3). A parallelism exists between this sodium loss and the appearance of dehydration, and undoubtedly these phenomena are closely related.

The occurrence of an augmented output of urinary sodium in dogs following suprarenalectomy was first reported by Loeb and his co-workers (4) who earlier described the lowering of the serum sodium concentration in patients during the crises of Addison's disease (5). They demonstrated the phenomenon in balance experiments upon animals whose urinary output was followed after operative removal of the glands, until their death in suprarenal insufficiency, and showed

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that the increased sodium excretion was usually accompanied by an increased volume of urine.

In a previous paper (3) we demonstrated, in agreement with Loeb (4) the fact that augmented loss of urinary water, relative to fluid intake, usually accompanied urinary sodium excretion in suprarenalectomized dogs following the withdrawal of the hormone injections. That such an increased urinary excretion of water, proportionate to its intake, occurs after withdrawal of fluids, was also subse-
suprarenalectomized dogs allowed free access to fluids, was also subsequently confirmed by Swingle and his coworkers (6). Nevertheless the relation of renal fluid loss to the dehydration which occurs during the course of cortical insufficiency is not clear. Further and more comprehensive study of the nitrogen and electrolyte metabolism, in its relation to the water balance, is necessary for the elucidation of this problem. For this purpose it is obviously necessary to avoid so far as possible the side effects due to circulatory failure which appear during the terminal stages, and to confine attention to the more immediate consequences of withdrawal and subsequent reinjection of the hormone in the suprarenalectomized animal.

EXPERIMENTAL

The total gain or loss of electrolytes, and the alterations in the volume of body water during the cycle of deficiency and recovery, as induced in the suprarenalectomized dog by withdrawal and subsequent reinjection of the cortical hormone, may be examined by means of metabolism studies in which the intake of water, nitrogen and salts is maintained at approximately constant values, and the only variable factor is the injection or withdrawal of the cortical hormone.

Most of the procedures employed for the conduct of the metabolism experiments reported in this paper have been previously detailed (3). Only adult male dogs were used. Methods for their preparation and care have been described. Fluids and sodium chloride (10 per cent solution) were given by stomach tube. The urinary bladder was catheterized and washed with 25 cc. of distilled water at the end of each period. The metabolism was determined by weighing the dog before and after each period, and the stool was rinsed and scrubbed with 2000 cc. or more of distilled water at the end of each period, which was also analyzed. The water content of the stool was ascertained. Owing to the nature of the diet these were always formed and excreted only at 2 to 4 day intervals. All samples of blood were obtained

arterial or cardiac puncture and collected anaerobically. They were replaced by the intravenous injection of equal amounts of normal saline solution. Heparinized plasma was used as previously described. Care was taken to have the plasma electrolyte and urea pattern, and the blood concentration, at a constant normal level throughout the control period prior to withdrawal of the cortical hormone injections. This was assured by estimations made 1 week previous to the beginning of the control period, on the same constant routine.

The columns in the tables headed water balance represent the excess or deficiency of total water intake in relation to total output as measured in the ingested food and fluids, and in the excreta, when compared to the control period, during which the weight and other factors considered were invariably constant.

It would be a great advantage if a synthetic diet, constant in composition, could be used in metabolic experiments on suprarenalectomized dogs. Unfortunately, it is extraordinarily difficult to induce such animals regularly, and over extended periods, to eat such rations, and particularly, to resume them during recovery from cortical insufficiency. We were therefore obliged, after the expenditure of much effort, to return to the use of lean meat (supplemented with calcium phosphate and cod liver oil, or with milk powder, when it was desirable to maintain the animals in calcium balance) as the simplest acceptable food. Each sample of meat had to be analyzed separately for its constituents. Nine complete balance studies have been made, without technical mishaps, the results of which are consistent. A number of other incomplete experiments in which vomiting or diarrhea, or death in insufficiency has resulted, have furnished, so far as it could be used, supplemental and concordant data.

The methods for chemical analysis have been previously described. The samples for sodium determinations (7) as well as for potassium determinations have been separately ashed in an electric muffle. Certain analytical details worked out with the assistance of Dr. Mary V. Buell render it possible to obtain consistent duplicate sodium analyses with an error of 1 per cent or less. The potassium method of Shohl and Bennett (8) has been used during the past year, and the recent rapid ashing method of Strauss by means of the addition of thorium nitrate has also been employed (9).

The urine specimens for potassium determination in the earlier experiments were prepared as follows: A suitable aliquot of the 24 hour specimen, both urine and cage washing, was measured into a 50 cc. pyrex beaker and evaporated to 1 cc. or less on the water bath. 10 cc. concentrated HNO_3 was added and the beaker covered and placed on the water bath for 4 to 7 days. The beaker was then uncovered and the sample taken to dryness. 10 cc. water plus 1 drop phenolphthalein was added and the solution was made alkaline with NaOH . The alkaline solution was evaporated to dryness (care being taken that this solution stayed alkaline while heating so as to drive off all ammonia). A few drops of water were added to the residue plus 5 cc. concentrated HNO_3 and the solution again taken to dryness and transferred quantitatively with water to a volumetric flask of suitable volume depending on the potassium concentration. The deter-

mination was continued as with the serum. More recently, as stated above, the rapid ashing method of Strauss with thorium nitrate has been employed.

Data from such experiments in which the intake of water is controlled at a normal level¹ or reduced (Tables I a, I b, I c) reveal that during the first phase of the suprarenal cycle, that following withdrawal of cortical extract, which is associated with increasing dehydration, the loss of sodium in the urine is accompanied by a retention of potassium. If the period of extract withdrawal is greatly prolonged and anorexia develops, potassium excretion is eventually increased (Table I b).

When the characteristic changes in the blood plasma have occurred as a result of the withdrawal of the hormone, and before any of the later effects of circulatory failure, diarrhea or anorexia appear, injections of the cortical hormone are again resumed. The phenomena of the recovery phase of the cycle consist of an immediate urinary excretion of potassium, accompanied by phosphates and urea, and retention, which is usually well marked, of sodium and chloride. The urinary excretion of potassium under the influence of the hormone during the early recovery phase is quite as striking as the excretion of sodium following extract withdrawal. When the fluid intake is not forced, a water diuresis takes place which is greater, and may, indeed, be several times the magnitude of that earlier induced by the cessation of the injections of cortical hormone. This is usually accompanied by a further loss of weight. The coincidental dilution of the blood plasma which generally occurs, is indicated by the diminished cell count and volume of packed cells. It should be pointed out that the electrolyte losses during the phase of insufficiency are chiefly sustained by the ions of principal concentration in the interstitial fluids, sodium and chloride, whereas the losses of the recovery phase are those characteristic of the cell structure, potassium and phosphate, together with nitrogen.

The loss of urinary potassium taking place as a result of the resumption of extract injections in suprarenal insufficiency cannot represent merely an augmented excretion resulting from anorexia. An experiment in which the animal was given neither potassium, nitrogen nor phosphate, still shows clearly the augmented excretion of

¹ The normal level of fluid intake on the constant diet was determined by actual measurement of the average fluid intake during a preliminary period of several days when it was freely offered.

TABLE 12

Effect of Withdrawal and Subsequent Reinjection of the Cortical Hormone

Dog 1-32. Balance experiment. Diet of 450 gm. raw beef, 300 cc. distilled water and 1 gm. sodium chloride per stomach tube daily. All figures calculated on a daily basis.

Date	Weight kg.	Food per day gm.	Cortical extract per day cc.	Water balance* cc.	Sodium balance m.eq.	Chloride balance m.eq.	Potas- sium balance m.eq.	Nitro- gen balance gm.	Urinary phos- phate output gm.	Plasma sodium per liter m.eq.	Plasma chloride per liter m.eq.	Plasma potas- sium per liter m.eq.	Blood urea mg. per 100 cc.	Red blood cells per c.mm.	Hemo- globin gm. per 100 cc.	Volume packed red blood cells per cent	Plasma pro- teins gm. per 100 cc.
1935																	
Mar. 5-8	10.6	450	8	—	-1.0	-0.6	+1.6	-0.1	0.65	144.8	111.1	4.3	20	4.9	9.8	36	6.0
				Extract withdrawn													
8-9	10.5	450	0	-263	-66.0	-50.7	+18.3	+0.3	0.53								
9-10	10.2	450	0	+52	-5.3	+1.6	+12.6	+2.9	0.49								
10-11	10.2	380	0	+35	-3.7	-10.9	+7.2	+4.4	0.33								
				Insufficiency. Extract resumed													
11-12	10.1	0	50	-510	-2.2	-9.2	-56.4	-13.2	0.82	133.0	101.5	10.6	140	7.0	14.4	52	6.9
12-13	9.6	100	50	-65	+16.0	+8.5	-15.9	-4.4	0.52	138.2	106.6	4.6	26				
13-14	9.7	150	15	+62	+16.1	+15.1	-1.2	-1.0	0.30								
14-15	9.7	270	15	+182	+23.3	+18.3	+16.5	+2.2	0.31								
15-16	9.7	390	10	+118	+20.5	+11.4	+11.8	+1.3	0.57								
16-18	9.9	450	10	+22	-10.0	+6.2	+7.8	+0.1	0.56								
18	10.1	450								140.5	107.4	4.9	16	5.4	10.2	37.2	6.4

* The water balance in each period is referred to the balance of the control period, Mar. 5 to 8.

TABLE 1b

Effect of Withdrawal and Subsequent Reinjection of the Cortical Hormone

Dog 1-51. Balance experiment. Diet of 450 gm. raw beef, 500 cc. distilled water and 2 gm. sodium chloride per stomach tube daily. The experiment was unduly prolonged, so that a negative potassium balance occurred as a result of anorexia during the latter part of the extract withdrawal phase. All figures calculated on a daily basis. The diuresis following extract withdrawal, associated with sodium loss, and that following resumption of extract, associated with potassium excretion and sodium retention, are clearly observed.

Date	Weight	Food per day	Cortical extract per day	Water balance*	Sodium balance	Potas- sium balance	Nitrogen balance	Plasma sodium per liter	Plasma potas- sium per liter	Blood non- protein nitrogen	Red blood cells per c.mm.	Hemo- globin	Volume packed red blood cells	Plasma pro- teins
1935	12.0	450	15	—	+0.2	+0.2	+2.1	145.5	4.6	34	5.31	11.2	34.5	6.8
		Extract withdrawn												
Apr. 27-30	11.9	450	0	-230	-51.6	-0.5	-2.0							
Apr. 30-May 1	11.9	450	0	-80	-13.4	+4.1	+0.8							
		450	0	-32	+3.2	+0.3	-0.1							
May 1-2	11.8	450	0	-130	-9.0	-6.3	-1.4							
2-3	11.8	350	0	-160	-6.7	-5.4	-1.0							
3-4	11.8	280	0	-157	-2.5	-5.4	-3.5							
4-5	11.8	140	0	-122	-5.4	-11.5	-3.0							
5-6	11.7	150	0		-8.6									
6-7	11.6	200	0											
7-8				Extract resumed	+20.8	-48.7	-6.4	128	8.8	70	7.3	15.8	51.4	7.4
				50	-415	+1.1	-0.6	137.4	5.1	36	4.2	11.8	35.0	6.2
8-9	11.2	450	36	-49	+21.7	+5.2	-1.7							
9-10	11.3	450	35	-160	-3.9	+12.5	+1.2	139.3	5.3	40	5.5	12.0	36.0	6.6
10-11	11.3	450	10	+40										
11-13	11.7	450												

Dog 1-51. Balance experiment prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. The experiment was unduly prolonged, so All figures calculated on basis of 100 gm. body weight. 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* The water balance in each period is referred to the balance of the control period, Apr. 27 to 30.

TABLE I C

Effect of Withdrawal and Subsequent Reinjection of the Cortical Hormone

Dog 1-57. Balance experiment. The animal ate 450 gm. raw beef daily throughout the entire experiment, except on Jan. 26 to 27 when it ate 330 gm. Constant water intake 500 cc. daily (by stomach tube), 4 gm. NaCl, 3 cc. cod liver oil and 0.5 gm. $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. On the days when extract was withdrawn 8 cc. of 0.85 per cent NaCl were substituted for injection of extract.

ection of extract.

Date	Weight	Cortical extract per day	Water balance*	Sodium balance	Chloride balance	Potas- sium balance	Nitro- gen balance	Urinary phos- phate output	Plasma sodium per liter	Plasma chloride per liter	Plasma potas- sium per liter	Blood urea	Red blood cells per cumm.	Hemo- globin	Volume packed red blood cells	Plasma proteins
1918	kg.	cc.	cc.	m.eq.	m.eq.	m.eq.	gm.	mg.	m.eq.	m.eq.	m.eq.	mg. per 100 cc.	millions	gm. per 100 cc.	per cent	gm. per 100 cc.
Jan. 19-22	13.8	8	—	+0.1	+1.1	+0.1	-0.3	675	138.8	101.6	5.8	20	5.9	12.2	38.8	6.9
			Extract withdrawn													
22-23	13.8	0	-250	-50.8	-40.6	+9.0	+1.0	473								
23-24	13.65	0	-75	-12.4	-1.7	+5.6	+1.5	538								
24-25	13.6	0	+20	-6.0	-19.1	+5.7	+4.4	496	132.4	91.6	12.3	105	6.5	13.0	41.0	6.9
			Extract resumed													
25-26	13.5	25	-442	-8.7	—	-50.2	-6.8	1049								
26-27	13.5	25	+1	+8.9	-0.6	-5.0		429	138.0	106.6	5.4	25	6.5	13.0	37.5	7.1
27-29		16	+15	+6.2	+0.4	-2.5	-1.3	656								
29-Feb. 1	13.8	8	+102	-2.4	-4.8	+3.1	+0.5	553	139.2		6.4	26	6.5	12.7	39.2	7.0

* The water balance in each period is referred to the balance of the control period, Jan. 19 to 22.

TABLE 1 b

Effect of Withdrawal and Subsequent Reinjection of the Cortical Hormone

Dog 1-51. Balance experiment. Diet of 450 gm. raw beef, 500 cc. distilled water and 2 gm. sodium chloride per stomach tube daily. The experiment was unduly prolonged, so that a negative potassium balance occurred as a result of anorexia during the latter part of the extract withdrawal phase. All figures calculated on a daily basis. The diuresis following extract withdrawal associated with sodium loss, and that following resumption of extract, associated with potassium excretion and

	Plasma	Blood non- protein	Red blood cell per cubic mm.	Hemo- globin	Volume packed red blood	Plasma pro- teins
--	--------	-----------------------	------------------------------------	-----------------	----------------------------------	-------------------------

[illegible]

** The water balance in each period is reported.

TABLE I C

Effect of Withdrawal and Subsequent Reinjection of the Cortical Hormone

Dog 1-57. Balance experiment. The animal ate 450 gm. raw beef daily throughout the entire experiment, except on Jan. 26 to 27 when it ate 330 gm. Constant water intake 500 cc. daily (by stomach tube), 4 gm. NaCl, 3 cc. cod liver oil and 0.5 gm. $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. On the days when extract was withdrawn 8 cc. of 0.85 per cent NaCl were substituted for injection of extract.

Date	Weight kg.	Cortical extract per day cc.	Water balance* cc.	Sodium balance m.eq.	Chloride balance m.eq.	Potas- sium balance m.eq.	Nitro- gen balance gm.	Ura- ny phos- phate output mg.	Plasma sodium per liter m.eq.	Plasma chloride per liter m.eq.	Plasma potas- sium per liter m.eq.	Blood urea mg. per 100 cc.	Red blood cells per c.mm. millions	Hemo- globin gm. per 100 cc.	Volume packed red blood cells per cent	Plasma proteins gm. per 100 cc.
1936																
Jan. 19-22	13.8	8	—	+0.1	+1.1	+0.1	-0.3	675	138.8	101.6	5.8	20	5.9	12.2	38.8	6.9
			Extract withdrawn													
22-23	13.8	0	-250	-50.8	-40.6	+9.0	+1.0	473								
23-24	13.65	0	-75	-12.4	-1.7	+5.6	+1.5	538								
24-25	13.6	0	+20	-6.0	-19.1	+5.7	+4.4	496	132.4	91.6	12.3	105	6.5	13.0	41.0	6.9
			Extract resumed													
25-26	13.5	25	-442	-8.7	—	-50.2	-6.8	1049								
26-27	13.5	25	+1	+8.9	-0.6	-5.0	—	429	138.0	106.6	5.4	25	6.5	13.0	37.5	7.1
27-29		16	+15	+6.2	+0.4	-2.5	-1.3	656								
29-Feb. 1	13.8	8	+102	-2.4	-4.8	+3.1	+0.5	553	139.2		6.4	26	6.5	12.7	39.2	7.0

* The water balance in each period is referred to the balance of the control period, Jan. 19 to 22.

potassium following resumption of cortical hormone (Table II). In this case the dog was in negative water, potassium and nitrogen balance throughout, but showed nevertheless the usual effects: in-

TABLE II

Balance Experiment, without Ingestion of Potassium, Phosphate or Nitrogen

Dog 1-45. Diet of 5 gm. glucose, 20 cc. cottonseed oil and 2 gm. sodium chloride (given by stomach tube). Water was offered freely but little was taken. The dog was in negative water, potassium and nitrogen balance throughout. Nevertheless the increased fluid loss on withdrawal and again on resumption of hormone, and the increased sodium loss on withdrawal and the sodium retention and increased potassium loss on resumption of the hormone injections on Jan. 14 to 15 are evident.

Date	Weight	Cortical extract per day	Water balance*	Sodium balance	Potassium balance	Nitrogen balance	Plasma sodium per liter	Plasma potassium per liter	Plasma nonprotein nitrogen	Red blood cells per c.mm.	Hemoglobin	Volume packed red blood cells	Proteins
1935	kg.	cc.	cc.	m.eq.	m.eq.	gm.	m.eq.	m.eq.	mg. per 100 cc.	mil-lions	gm. per 100 cc.	per cent	gm. per 100 cc.
Jan. 5-7	8.9	8	—	+1.2	-10.2	-3.9	140.6	4.9	34	4.6	9.5	33.0	6.7
			Extract withdrawn										
7-8	8.6	0	-280	-19.2	-10.6	-2.3							
8-9	8.4	0	-197	-3.5	-5.3	-3.4							
9-10	8.3	0	-237	-14.9	-4.7	-2.5							
10-11	8.1	0	-250	-10.8	-5.3	-2.2							
11-12	7.8	0	-202	-15.7	-6.1	-2.1							
12-13	7.7	0	-215	+6.6	-5.8	-1.3							
13-14	7.6	0	-140	+3.6	-5.3	-1.0	130.4	7.5	68	5.6	11.8	42.0	7.0
			Extract resumed										
Animal in insufficiency.													
14-15	7.4	40	-220	+0.3	-20.0	-2.8							
15-16	7.4	40	-215	+11.4	-16.6	-2.7							
16-17	7.4	40	-30	+20.8	-4.2	-1.7	135.8	3.9	32	3.7	8.0	26.0	6.1

* The water balance is referred in each period to the balance of the control period, Jan. 5 to 7.

creased fluid loss on withdrawal and again on resumption of hormone injections, increased sodium loss on withdrawal, sodium retention on resumption, and finally, marked potassium loss on resumption of

extract injections. Experiments have also been carried out in which the food intake was constant while the output of potassium and nitrogen was still characteristically increased (Table I c). During the recovery phase both the total output and the concentration of potassium are increased.

TABLE III

Dog 1-59. Balance experiment, similar to that shown in Table II except with lower salt intake and higher water intake per kilo body weight. Diet of 450 gm. raw beef with 1 gm. sodium chloride and 500 cc. distilled water by stomach tube daily. With such a large amount of fluid in proportion to salt intake no diuresis is observed and a remarkably constant water balance is noted, although the changes in sodium and potassium associated with withdrawal and injection of the hormone are clearly observed.

Date	Weight	Food per day	Extract per day	Water balance*	Sodium balance	Potassium balance	Nitrogen balance	Plasma sodium per liter	Plasma potassium per liter	Plasma nonprotein nitrogen	Red blood cells per c.mm.	Hemoglobin	Volume packed red blood cells
1935	kg.	gm.	cc.	cc.	m.eq.	m.eq.	gm.	m.eq.	m.eq.	mg. per 100 cc.	millions	gm. per 100 cc.	per cent
Sept. 27-Oct. 1	9.5	450	10	—	+0.9	+1.0	-0.2	142.6	4.9	31	4.5	10.4	30
					Extract withdrawn								
Oct. 1-2	9.4	450	0	-47	-39.6	+16.1	+1.1						
2-3	9.4	450	0	-14	-30.5	+9.0	+0.8						
3-4	9.25	450	0	+73	-11.3	-4.1	+2.2	129.6	9.7	116	5.4	13.0	40.3
					Extract resumed								
4-5	9.3	450	50	-11	+20.0	-25.5	-4.3	135.8	5.8	48	4.6	11.5	37.0
5-7	9.4	450	30	+34	+2.9	-2.3	-0.5						
7-9	9.4	450	15	—		-2.7	+0.3	139.6	5.7	46	4.7	10.4	34.6

* The water balance is referred in each period to the balance of the control period, Sept. 27 to Oct. 1.

It is possible, however, to show that diuresis is not an essential consequence either of withdrawal or of exhibition of the hormone (Table III). If sufficient fluid is forced by mouth, and the salt intake is kept low, little or no diuresis is observed, and the weight loss is in-

considerable. Nevertheless, the characteristic electrolyte changes and hemoconcentration take place, and if salt or cortical extract are not resumed in time, the animal will die. Water alone or glucose solution will not delay greatly the onset of suprarenal insufficiency in the dog under these constant conditions of food and sodium chloride intake.

The occurrence of diuresis during the cortical deficiency cycle, therefore, seems to depend largely upon the amounts of salt ingested in relation to the consumption of water. The dog in confinement, when offered fluids freely, ingests amounts which are fairly closely conditioned by its food intake. Where water in sufficient amount in proportion to salt is not forthcoming, the supplies of endogenous water are levied upon for the formation of urine. It is a striking fact, despite the marked evidences of dehydration and shock which occur in experimental suprarenal insufficiency in the dog, and which are to be observed clinically in the crises of Addison's disease, that thirst is never conspicuous, and when food is refused, water is refused as well. It is in contrast to the desire for water found in other forms of shock, as, for example, after extensive hemorrhage.

DISCUSSION

The excretion of potassium and of nitrogen during the recovery from suprarenal insufficiency has been shown, in these experiments, to be considerably greater than can be accounted for by the apparent accumulation in the plasma and interstitial fluids (Table IV, column 5). The normal quantity of intracellular fluid is believed, as a first approximation, to be from two and one-half to three times that contained in the interstitial fluid (10), while the interstitial fluid of the body is said to represent about 20-25 per cent of its weight. With the contraction in volume sustained by the interstitial fluids, including the plasma, during the phase of insufficiency, and the probable simultaneous increase in intracellular fluids (10, 11), the ratio must be still greater. It seems very unlikely that the loss of potassium, phosphate and nitrogen following the readministration of the hormone represents a coincidental destruction of cellular tissue. On the contrary, a marked improvement appears at once in muscular function, inconsistent with a theory of destruction of protoplasm (12). The observed excretion of potassium in most experiments is approximately the amount which would be

discharged if the potassium level throughout all of the body fluids were lowered to about the same extent as is its concentration in the plasma. A much greater removal of potassium than that ascribable to tissue destruction has been reported in a variety of conditions and the fact is quite clearly established.

The following working hypothesis is proposed to fit together the observations thus far established in regard to water, sodium and potassium movement during the cycle of cortical insufficiency and

TABLE IV

Comparison of the Potassium Loss from the Interstitial Fluids with the Total Potassium Loss Following Resumption of Cortical Hormone Injections

Experiment	1 Approximate sodium loss during 48 hrs. after withdrawal of cortical extract	2 Approximate potassium loss during 48 hrs. after resumption of cortical extract	3 Coincidental fall in plasma potassium concentration per liter	4 Total excretion of potassium from interstitial fluids assuming them to represent 25 per cent of the body weight	5 Loss of potassium in excess of estimated potassium loss from the interstitial fluid
	<i>m.eq.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>m. eq.</i>	<i>m. eq.</i>
Ia—Dog 1-32	71.3	72.3	6.0	18.1	54.2
Ib—Dog 1-51	65.0	49.8	3.7	10.7	39.1
Ic—Dog 1-57	63.2	55.2	6.9	23.3	31.9
III—Dog 1-59	70.1	27.8	3.9	8.8	19.0

Column 4 indicates the fall in plasma potassium concentration per liter multiplied by 25 per cent of the body weight, producing an approximation of the potassium concentration in the total volume of extracellular fluid.

Column 5 represents column 2 minus column 4.

recovery. We assume that the effect of the cortical hormone regulating water and salt is exerted upon the renal excretion of potassium and sodium. In its absence, under ordinary conditions of salt and water intake, sodium is excreted in abnormal amount and potassium is retained. During the process of recovery the reverse condition occurs; sodium is retained and potassium is excreted until the proper plasma levels are restored. Not only is the total potassium increased, but its urinary concentration is raised as well.

We propose further to assume that potassium is present in the cells in both a nondiffusible form, which may be in closer chemical or

physical relationship to the cellular proteins, and in a diffusible form which is normally present in approximately the same low concentration in which it occurs in the extracellular fluids. With the loss of urinary sodium derived from the interstitial fluids, occurring on removal of the regulatory influence of the cortical hormone, water enters the tissue cells (10, 11). It is possible that this disturbance of cellular equilibrium in turn causes a portion of the nondiffusible potassium moiety to break down into the diffusible form, which may then pass out into the extracellular fluids until the concentration is approximately the same throughout all of the body water. Under ordinary circumstances the kidneys would excrete this excess at once, but in the absence of the cortical hormone there is renal retention of potassium and the concentration in the body fluids continues to rise as insufficiency progresses.

Upon resumption of the hormone injections, however, the renal barrier to potassium excretion is removed and the excess is excreted by the kidney, both from the extracellular fluids and from the diffusible potassium of the intracellular fluids as well, carrying with it the excessive accumulation of intracellular water. A large portion of the water so discharged from the cells, however, is retained in the interstitial fluid and plasma, since the kidney is again able to excrete urinary potassium at its normally high concentration. The actual amount of potassium which is excreted, namely two and one-half to five times the estimated loss from the extracellular fluids (Table IV) is approximately the quantity which should be discharged on this assumption.

The rise in the concentration of blood nonprotein nitrogen, which is chiefly, but not exclusively, in the urea fraction, during the course of suprarenal insufficiency following withdrawal of extract from these dogs, both in these experiments and in those previously reported (13) is more closely related to the rise in plasma potassium than it is to the fall in plasma sodium or chlorides. Kerpel-Fronius and Butler (14) have assembled data of their own and from the literature indicating the lack of necessary association between electrolyte concentration and azotemia, and similar data may be found in suprarenal insufficiency in the dog with alterations in salt and water intake (13). It is to these variations, we believe, in salt and water intake and excretion, that the

increased sodium and chloride levels in the plasma may be attributed which have been described during suprarenal insufficiency in the marmot and opossum (15). Early in suprarenal insufficiency there appears to be an actual reduction of urea clearance (12)² which precedes both dehydration and fall in blood pressure, occurring within a day or two of extract withdrawal.

The consequences of withdrawal of suprarenal cortical extract in the suprarenalectomized dog may be compared to those produced by certain diuretic agents. They differ from the effects of diuretin diuresis, as reported by Kerpel-Fronius and Butler (14), in which a marked excretion of potassium, as well as of sodium, chlorides and nitrogen takes place. Loss of potassium is also reported to follow the use of acid-producing diuretics in human subjects (16).

The data herein presented show that hormone withdrawal characteristically produces a selective loss of urinary sodium and chloride without increased urinary withdrawal of nitrogen and potassium, and that renal excretion of water in excess of that lost in the control periods when the animal is sustained with adequate injections of the cortical hormone, is largely conditioned by the requirements of urinary sodium excretion. If the water intake is adequate to carry away the sodium, no fluid need be withdrawn from the intrinsic body stores. Water loss is therefore secondary to loss of salt. On the other hand, the injection of cortical extract during insufficiency does often produce diuresis associated with the loss of potassium, nitrogen and phosphate, but this is accompanied by no characteristic immediate increase in sodium and chloride excretion. Again, it is clearly not analogous to the effects of the diuretic agents mentioned above.

The effect of cortical hormone injections upon the urinary excretion of potassium in suprarenal insufficiency may be compared to the effect of pituitrin upon the excretion of electrolytes in the dog. Stehle (17), and McIntyre and Sievers (18), have demonstrated an increased excretion of potassium, which was augmented to a much greater degree

² Our own studies on urea clearance following withdrawal of extract from the suprarenalectomized dog confirm this observation by Loeb (12), as well as the rapid return in the clearance following resumption of hormone injections. These variations in urea clearance were noted while food intake was unimpaired and before changes in the circulation were detectable.

than sodium, after the injection of this hormone. These were in experiments of short duration. McIntyre and Sievers confirmed the constant rise in the potassium-sodium ratio in the urine after pituitrin, but at the same time found no alterations in the serum concentrations of these electrolytes. Smith and MacKay (19) have reported a study of the effects of repeated injections of surgical pituitrin (0.5 cc. twice daily) upon a normal man. The changes found in the balance of the several ions are somewhat similar to the results effected by cortical extract injection in the suprarenalectomized dog. The influence of the diuretic principle of the anterior lobe as described by Teel (20), and subsequently by others, is not yet clear. Further studies are in progress in an attempt to elucidate the relation existing between the salt and water function of the suprarenal cortex and the antidiuretic and diuretic principles of the posterior and anterior lobes of the pituitary gland.

The data presented offer further evidence that the conspicuous effect of the cortical hormone is upon the renal excretion of the electrolytes sodium and potassium, and that changes in water excretion are secondary to this primary action. Stress should be laid upon the short duration of the potassium diuresis when effective hormone therapy is given. It lasts but 24 or at most 48 hours. If the period of observation were extended over 2 or 3 days, using other laboratory animals such as the rat, where exact urinary excretion with respect to time may not be readily measured, this phenomenon might be completely masked.

The dilution of the blood plasma which constantly follows injection of the hormone during the state of cortical deficiency, is usually well marked, whether diuresis also occurs or not. Such a dilution also follows the injection of pituitrin or pitressin, as Yanagi has again recently emphasized (21). It is frequently surmised that changes in plasma volume reflect a general change of similar magnitude in the total volume of interstitial water. We do not know that this necessarily follows. The increased potassium excretion doubtless comes from the cellular structures. The suggestion that pitressin increases the cellular permeability for potassium and water (22) might hold with equal plausibility for the effect of the cortical hormone when it is given in suprarenal insufficiency. The balance of evidence, we believe, is against this assumption.

TABLE V

Effect of Sodium Chloride Alone on Animal in Suprarenal Deficiency

Dog 1-32. Balance experiment. Diet of 450 gm. raw beef + 500 cc. distilled water + 1 gm. sodium chloride by stomach tube daily. After 5 day control period on extract, withdrawal produced the usual electrolyte and water balance changes. On the 3rd day after extract withdrawal, May 20, the animal was given 6 gm. sodium chloride by mouth (fluid intake unchanged). It vomited slightly during the night which rendered it necessary to discard the electrolyte excretion for May 20 to 21. On the morning of May 21 the plasma electrolyte concentrations were approaching normal values and slight hemodilution had occurred, but the animal was very ill and required extract. There was no loss of weight nor diuresis. This produced fluid retention in place of the usual diuresis.

Date	Weight	Food per day	Urine output	Cortical extract per day	Water balance*	Sodium balance	Potas- sium balance	Nitrogen balance	Plasma sodium per liter	Plasma potassium per liter	Plasma non-protein nitrogen	Plasma proteins	Red blood cells per c. mm.	Hemo- globin	Volume packed red blood cells
	kg.	gm.	cc.	cc.	cc.	m.eq.	m.eq.	gm.	m.eq.	m.eq.	mg. per 100 cc.	gm. per 100 cc.	millions	gm. per 100 cc.	per cent
May 13-18	10.2	450	633	10		+0.3	+0.8	+0.4	138.3	5.2	36	6.0	5.0	12.3	35.7
18-19	9.9	450	850	0	-223	-53.7	+10.0	+1.2							
19-20	9.9	450	600	0	+27	-5.4	+6.0	+1.4							
20-21	9.9	Given 6 gm. sodium chloride by mouth 10 a.m., May 20 with additional fluid													
		450	570	0					132.6	11.1	85	7.5	7.9	16.2	50.3
		Dog is ill. Walks only with stagger. Diarrhea and vomiting. Specimens discarded.													
21-22	9.7	350	460	50	+142	+22.3	-20.2	-0.8	141.7	6.8	70	6.9	6.9	15.2	45.3
22-23	10.0	450	540	25	+110	+17.0	+0.2	+0.5							
23-25	10.1	450	587	10	+50	-0.6	+5.3	+0.2							
									141.0	4.7	32	6.4	4.3	8.2	27.5

* The water balance in each period is referred to the balance of the control period.

* The water balance in each period is referred to the balance of the control period, May 13 to 18.

TABLE VI
Effect of Added Water Alone (5 Per Cent Glucose Solution) on Animal in Suprarenal Deficiency

Dog 1-32. Balance experiment. Diet of 450 gm. raw beef, 500 cc. distilled water + 1 gm. sodium chloride by stomach tube daily. Cortical extract was withdrawn during 2 days (Apr. 28 to 29 and 29 to 30) with the usual effect upon sodium and potassium balance. On the 3rd day (Apr. 30 to May 1), the animal was given in addition to the constant food and fluid intake by mouth, 235 cc. 5 per cent glucose intravenously, which caused a fluid retention and hemodilution, but with an aggravation of the plasma electrolyte changes characteristic of insufficiency. Cortical extract the following day produced the usual effects upon water balance, sodium, potassium and nitrogen. The urine for Apr. 30 to May 1 was discarded because the animal vomited.

Date	Weight		Food		Cortical extract		Water balance		Sodium balance		Potassium balance		Nitrogen balance		Plasma sodium per liter		Plasma potassium per liter		Plasma non-protein nitrogen		Plasma glucose		Plasma phosphates		Red blood cells		Hemoglobin		Volume packed red blood cells		Plasma proteins	
	kg.		gm.		cc.		cc.		m.eq.		m.eq.		m.eq.		gm.		m.eq.		m.eq.		mg. per 100 cc.		mg. per 100 cc.		millions		gm. per 100 cc.		per cent		gm. per 100 cc.	
1935																																
Apr. 25-28	10.4		450	15		-2.8	-0.5	+0.8	143.9	5.4	40	85	3.2	5.1	11.0	36.3	6.4															
				Extract withdrawn		-90	-25.0	+3.2	+0.1																							
				0		+32	-19.5	+8.7	+1.3	7.9	72	87	7.0	6.3	13.3	43	7.1															
28-29	10.2		450	0																												
29-30	10.3		450	0																												
Apr. 30-May 1	10.4		200																													
				0		Vomited in cage.	Specimen had to be discarded	130.6	12.9	108	80																					
May 1-2																																
						Extract resumed																										
				50		-443	+16.8	-30.3	-6.0																							
				40		-18	+17.6	-2.2	-0.5																							
2-3	10.1		280	40		+146	+1.0	+7.9	+1.3	135.0	6.1	44	110	3.6	4.4	9.6	33.4	6.7														
3-4	10.3		400	10																												

The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. On the 1st day, 235 cc. 5 per cent glucose intravenously. The urine for Apr. 30 was cloudy. 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* The water balance in each period is referred to the balance of the control period, Apr. 25 to 28.

The possibility of therapeutic application of these findings to the treatment of Addison's disease has not been overlooked, but we believe that attempts at potassium regulation clinically are of uncertain practical importance. High values for serum potassium concentration are observed in this condition, but when present usually occur only terminally. Balance studies during this stage of the disease have been impossible because of the vomiting and diarrhea which commonly occur, and because of the therapeutic requirements which demand the administration of large amounts of fluids and sodium chloride. The probable rôle of the phenomena observed in suprarrenal cortical insufficiency in the dog, such as cardiac irregularities and bradycardia, has been pointed out previously (23).

It is possible, by similar balance experiments, to contrast the effect, (a) of administration of sodium chloride alone (Table V), or (b) of added water alone, injected as 5 per cent glucose solution (Table VI), with that of the cortical hormone alone, in suprarenalectomized dogs. Neither study is quite complete. In the experiment in which sodium chloride without extra fluid was given, diarrhea and vomiting occurred during one 24 hour period. In the experiment with injection of intravenous glucose the urine specimens had to be discarded for 1 day because contaminated with a small quantity of vomitus. Nevertheless, it is clearly evident that the ingestion of dry sodium chloride by mouth, without increasing the fluid intake, was effective in restoring within 24 hours a more nearly normal plasma electrolyte pattern, and in diluting the blood. On the other hand, the intravenous infusion of glucose had no such effect, the distortion of the plasma electrolyte pattern becoming still more prominent, although a considerable dilution of the blood also did occur. In both cases, the injection of cortical extract was necessary in order to save the animal, and it produced the usual effects upon the balances of sodium and potassium.

SUMMARY

1. The withdrawal of maintenance injections of the cortical hormone from the suprarenalectomized dog during balance experiments, in which a constant meat diet is given, with constant fluid and salt intake, is followed by increased urinary loss of sodium and chloride, and by retention of potassium and nitrogen.

2. Where the water intake is low, a definite diuresis usually accompanies this excretion of sodium and chloride, but where fluids are forced, no diuresis may be observed.

3. The reinjection of the cortical hormone in suprarenal insufficiency causes an active renal excretion of potassium which is greatly in excess of the probable extra accumulation of this component in the extracellular fluids during the period when insufficiency is developing. This potassium excretion is surmised to be sufficient to account for such an accumulation, if diffusible potassium is present in like concentration equally throughout all of the body water, intracellular as well as extracellular. The excretion of potassium is accompanied by a corresponding excretion of phosphate and of nitrogen.

4. The excretion of electrolytes which is associated with withdrawal and with subsequent reinjection of suprarenal cortical hormone differs from the effects produced with various diuretic agents regarding which data are available. The effects produced by injection of the cortical hormone³ during suprarenal insufficiency, however, do resemble those produced with pituitrin, particularly in the greatly increased excretion of potassium relative to sodium, and in the coincidental dilution of the circulating blood. They suggest the possibility that the two similar effects may be ascribable to a common cause.

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³ In another place (24), we have suggested that the term "water and salt" hormone of the adrenal cortex might be a more appropriate designation for this substance. It was first employed by Long and Lukens (25), and has the advantage that it avoids confusion with other physiological activities ascribed to the cortex, and leaves clearly as an open question the possibility of other hormonal principles in this gland which may or may not be related.

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CHANGES IN OUTLYING BONE MARROW ACCOMPANYING
A LOCAL INCREASE OF TEMPERATURE WITHIN
PHYSIOLOGICAL LIMITS*

BY CHARLES HUGGINS, M.D., AND B. H. BLOCKSOM, JR., M.D.

(From the Department of Surgery, The University of Chicago, Chicago)

PLATES 13 TO 16

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This report is concerned with the functional reasons for the distribution of red and yellow bone marrow in the extremities of mammals and birds. Bone marrow is largely confined to the interior of the skeleton in these species. Exceptions are the physiological occurrence of hemopoietic tissue in the liver and spleen during fetal and neonatal life and in certain diseases such as myelogenous leukemia and the rare heterotopic hemopoietic foci seen in the adrenal gland (1), pelvis (31), and elsewhere. The reasons for this predilection of blood cell forming tissue for cavities in bone are still unknown. In the production of bone by ligation of the renal pedicle in rabbits, caused by the action of renal pelvis epithelium on adjacent fibroblasts (2), hemopoietic marrow is observed (3).

At birth, in mammals and birds, all of the bones which contain marrow contain red marrow (4, 6), which is similar in all loci, suggesting that the erythropoietic stimuli are of a general nature; in an examination of more than 50 newborn mammals we found no exception. Soon after birth, a replacement of red marrow by fat takes place, greater in certain regions than in others, but present in normal animals in all bones. In adult life, marrow containing enough chromogens to produce a red color is found in skull, clavicles, scapulae, sternum, ribs, vertebrae, pelvis, and in the proximal portions of the extremities,

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and fat is present in these areas, mostly in amounts only visible microscopically. This fat is associated in part with a surplus of blood forming tissue in the normal organism at sea level, since under a low O_2 tension the fat recedes and marrow becomes hyperplastic in these central areas. A different situation exists in the distal regions of the extremities, where blood cell formation stops and the bone marrow is yellow with fat in the gross. An examination of the literature shows topographical variations in normal distribution in the extremities which is the subject of the present investigation.

The Distribution of Fatty and Red Bone Marrow in the Extremities of Normal Animals

Exact data on the anatomical distribution of fatty and hemopoietic marrow are not extensive. Certain technical difficulties are potentially present in that sawing of bones is apt to distribute blood-stained bone dust over the surface of yellow marrow, and secondly that unless the bones are examined soon after death hemolysis and staining of yellow marrow from gravitation of blood pigment and infiltration is apt to occur; both difficulties are surmounted by histological examination.

The evidence substantiates the observations of Neumann (5) concerning the centripetal regression of red bone marrow in these regions. The data in mammals may be summarized as follows.

Man: Neumann (5) in his classical contribution to the subject reported: (a) In none of the cases (mostly adults) was there found yellow marrow in the shaft of long bones with red marrow in both epiphyses. (b) Either the bones of the extremities contain fatty marrow exclusively or red marrow is confined to the upper parts of the arm and thigh bones (upper epiphysis and more or less of the adjacent diaphysis) while the more peripheral bones contain only yellow marrow. (c) Yellow marrow occurs in a centripetal manner and marrow hyperplasia after hemorrhage develops centrifugally. (d) There is a congruence with respect to yellow marrow distribution in upper and lower extremities. Piney (6) examined the marrow distribution in 91 subjects between the ages of 3 days and 83 years. At birth the marrow had a rich red color. At age 12 to 14 years fatty metamorphosis was complete in tibia, fibula, and lower femur, but not in proximal femur; this is the adult condition. In tarsal and carpal bones, fatty metamorphosis was complete before the whole of the diaphysis of bones, fatty metamorphosis was complete before the whole of the diaphysis of

tibia, fibula, radius, and ulna was fatty. Litten and Orth (7), Hedinger (11), Askanazy (4), and Fahr (8) reported on the adult femur marrow and agreed that in the majority of cases the red marrow in this bone is localized in the proximal end and that it is unusual to find the proximal end yellow and the distal end red. Peabody (9) reported that the marrow of the long bones is normally in greater part fatty and hypoplastic as opposed to the active extremely complex marrow of vertebrae and flat bones. Williams (28) studied the bone marrow from lumbar vertebrae, sternum, and junction of the lower and middle thirds of the humerus, femur, and tibia in 100 unselected necropsies in adults, and found that red marrow occurs in the long bones in orderly combinations. When hyperplasia was present in tibia it was also present in femur and humerus; this combination occurred in 2 cases. In 31 cases hyperplasia was present only in femur and humerus. In 25 cases hyperplasia was present in humerus alone.

Horse: Ackerknecht (10) examined the bone marrow of 120 horses of various ages and states of nutrition and found that red marrow persisted throughout life in the proximal and middle thirds of the femur with some variation in pattern. Red marrow was not found except in disease, in the radius, tibia, and more distal bones after 3 years of age. In the series of pathological horses the proximal epiphysis of tibia contained red marrow 4 times, the distal never. *Dog:* Oehlbeck, Robschelt-Robbins, and Whipple (12) studied the red marrow spread in normal dogs and found that in young animals it occupied nearly all the cancellous bone in ribs, vertebrae, and long bones. As the dog matured, red marrow occupied all of the cancellous bone in ribs and vertebrae but much less in the long bones, where femora and humeri might show a third of the marrow cavity filled with fat instead of red marrow, while tibiae, radii, and ulnae were two-thirds fatty. Bock (13) stated that the marrow is similar to other great mammals as found in the horse (10).

In the *pigeon*, Muller (14) reported that radius marrow is moderately fatty and femur is usually red, and Doan, Cunningham, and Sabin (15) stated that the bone marrow of the radius and ulna in the normal adult pigeon was similar to normal mammalian marrow of long bones.

The present authors sawed the femur and tibia of 17 mature normal dogs and found: (a) In all cases there was yellow bone marrow corresponding to the epiphyses of the femur and tibia bordering on the knee joint. (b) Femur: all dogs had red marrow in this bone; in 6 it was completely red as far as the knee epiphysis; in 10 there was a yellow island of fat occupying the middle third of the femur surrounded by a red peripheral bark and with red marrow above and below; in 1 the red marrow occupied the proximal third of this bone and all distal bones had fatty marrow. (c) Tibia: 1 dog had completely red marrow except at the epiphyses; 5 had completely fatty marrow; 8 had red

marrow in the proximal quarter to third of the diaphysis; 3 had red marrow in proximal and distal areas of metaphysis with a fatty island in the middle third. *Rabbit*: In this laboratory in 8 adult rabbits the marrow of pelvis, femur, and the proximal two-thirds of the tibia was found to be very red; rather abruptly a change took place in the distal third of the tibia where the marrow changed from red hemopoietic marrow to yellow marrow, nearly or quite fatty. The metatarsals were always yellow marrow, grown rabbits. It was found possible to recognize topography from microscopic sections of the marrow from the proximal and distal regions of the tibia in adults judging purely from fat content. *Albino rat*: A situation similar to the rabbit was found in 40 adult animals in this laboratory; the marrow of the leg was always red as far as the proximal two-thirds of the tibia; the metatarsal marrow was always fatty.

It will thus be seen that the distribution of red marrow spread in the extremities is not entirely an anatomical problem of fatty diaphysis and red epiphysis as will be discussed below, and it is certainly not one wholly of age as was early demonstrated by Litten and Orth (7). The evidence clearly shows that fat accumulates in the limbs in a centrifugal direction with a corresponding decrease of hemopoietic tissue.

No one save Whipple and his associates has offered an opinion as to the reasons for red-yellow marrow distribution. These investigators (12) felt that the red marrow does not expand as fast as does the mass of cancellous bone in the growing skeleton and that the liver might be the limiting factor in red marrow spread in setting the top limit for maximal red cell and hemoglobin production. Piney (6) did not believe that the proximal-distal variations of marrow distribution could be explained by gravity. Ackerknecht postulated that the centripetal development of fat was a physiological process and Askanazy referred to it as a secondary physiological lipomatous atrophy or involution.

McMaster and Haessler (32) studied the marrow in rabbits rendered anemic from blood loss, and found a greater red marrow spread in a group where a concentrated hemoglobin solution was injected intramuscularly than in uninjected animals.

An important observation was made by Ranvier (16) that the vertebrae of the tail of adult cattle, dog, and rabbit contain yellow fatty marrow. This was found true for more than 100 normal adult albino rats examined in this laboratory. In this animal all of the trunk verte-

brae have lateral bony processes and contain red marrow. Yellow marrow begins in the tail, one or two vertebrae beyond the last trunk segment equipped with a lateral process, and extends to the tip in adults. The transition is exceedingly sharp, indeed often in the proximal end of one vertebra the marrow will be mostly red, while the distal end contains mostly fat and all distal vertebral marrows are fatty.

The problem may then be posed: Why is bone marrow in the distal region of limbs and tail at a disadvantage from the standpoint of formation of blood as compared with marrow in the bones of the body trunk?

The sharpness of transition so frequently observed between red and yellow marrow suggests that a physical agent is the responsible factor. One of the fundamental differences between proximal and distal tissues is the higher temperature of the former. It has recently been shown (29) that the bone marrow participates in the thermal decrement occurring in the extremities. The following experiments were devised to learn if an increase of temperature would affect the distribution of red and yellow marrow in the extremity. Since the rat tail is always fatty after an early age, most of the present experiments were done on this structure which was found to serve as an excellent indicator for red marrow spread.

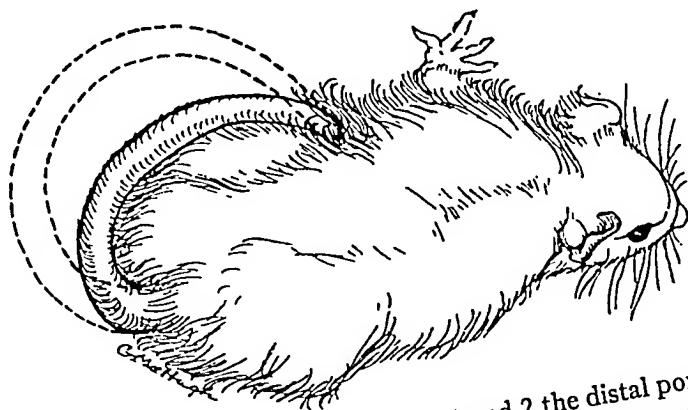
Effect of an Increase of Temperature on the Intact Adult Rat Tail

One of the simplest methods found to produce a chronic elevation in temperature in bone marrow is to denude the skin and surgically anastomose the otherwise intact structure to the abdominal wall so that the tail is inserted and maintained in the peritoneal cavity (Text-fig. 1).

Series 1.—Under ether anesthesia,¹ 35 albino rats between 27 and 200 days of age were operated upon aseptically. The skin of the tail was circumcised at about its middle and the distal skin removed and discarded. A 1 cm. incision was then made in the lateral abdominal wall and the denuded tail inserted into the peritoneal cavity and anchored by a suture on either side between a longitudinal tendon bundle and the loin muscles. The skin of the tail was then sutured at its cut margin to the abdominal incision. No dressing was applied and it was found that

¹ All operations were performed under ether anesthesia.

prompt healing usually took place leaving an *outside loop* to be compared as a control area with the intra-abdominal portion. In some of the experiments a few terminal segments were amputated for additional control observations; this did not appreciably affect the experiment. In all of the present experiments the tail was permitted to follow a gentle curve from sacrum to peritoneal cavity to avoid the effects of sharp angulation. The animals were killed at 5 to 7 day intervals between the ages of 17 and 111 days. At autopsy a part of the outside loop was compared with the intra-abdominal portion. All of the histological preparations described in this report were made from paraffin sections of tissue fixed in Zenker's fluid with 10 per cent formalin added, and decalcified in 5 per cent nitric acid.



TEXT-FIG. 1. In the experiments of series 1 and 2 the distal portion of the tail was skinned and surgically inserted in the abdomen, forming an outside tail loop. Differences in effect were observed when the outside loop was short or long (dotted lines).

An approximately similar experiment devised for a different purpose was reported by Ribbert (17), Matsuoka (18), Müller (19), and Mau (20, 21). These investigators studying the problem of spinal curvature denuded the skin of the tail in rabbit or rat and anchored it beneath the skin of the trunk to produce marked angulation of the bones. The effect of this procedure on the bone marrow was not reported.

It was thus possible to produce a chronic elevation of temperature of the distal intraperitoneal portion of the tail and to compare it with a more proximal outside loop of the same tail at a lower temperature without compromising blood, nerve, or lymphatic supply; the excised tip, moreover, in certain experiments served as further control tissue. Distinct changes in the bone marrow were observed as a result of this

operative procedure. In each instance, increased hemopoiesis was found in the segment of increased temperature as compared with the outside loop. In two experiments of the shortest duration the increase was slight, but in all the others obvious and unequivocal. More striking results were obtained in animals operated on between the ages of 27 to 50 days than in the oldest animals.

Several typical protocols will be given; the initial figures in each case signify the laboratory number, the age at the beginning, and the duration of the experiment.

Rat 8-78, old rat, 17 days: *Intra-abdominal portion* shows wide dilatation of blood vessels, increase of protein framework, and slight clumping (+) of hemopoietic cells in metaphyseal regions. *Outside loop*: normal vessels, very rarely a small cluster of cells is seen.

Rat 8-85, old rat, 38 days: *Intra-abdominal portion* (Fig. 3) shows a much greater dilatation of vessels and moderate hemopoiesis (++) with many red and white cell precursors, and megacaryocytes as compared with the almost completely fatty *outside loop*.

Rat 8-88, old rat, 65 days: *Intra-abdominal portion* shows much gelatinous protein material and vascular dilatation with moderate hemopoiesis (+), especially in the neighborhood of the metaphyses; the *outside loop* shows less gelatinous groundwork, slight capillary dilatation, no hemopoiesis.

Rat 8-98, 28 days, 46 days: *Intra-abdominal portion* shows an increase of protein framework in the diaphysis, with moderate hemopoiesis (+++) in the metaphyseal areas; *outside loop* is very fatty and has no hemopoietic foci.

Rat 8-80, old rat, 111 days, weight 260 gm.: *Intra-abdominal portion* shows hemopoiesis (++) with many red cell precursors; *outside loop* contains only normal fatty marrow without hemopoiesis.

Rat B, 27 days, 86 days: *Intra-abdominal portion*, much hemopoiesis (+++), many megacaryocytes, slight vascular dilatation (Fig. 1); *outside loop*, entirely fatty, no sign of hemopoiesis anywhere in the sections (Fig. 2).

An increase of vascularity, protein framework, and hemopoiesis was seen in that portion of the rat's tail which had been subjected to a chronic elevation of temperature, both as compared with the cooler outside loop and the normal tail. In most of the outside loop segments, the normal fatty marrow of the adult rat tail was seen; in some, however, there were observed increased framework and vascularity, with slight hemopoiesis; in no case did the magnitude of these changes approach that of the inside portion. Variations in temperature of the outside loop occurred depending on whether a small or large portion

of the tail was in the peritoneal cavity. In the former case, the long redundant loop was cooler than when this structure was short and closely applied to the trunk and thus influenced by apposition to warm structure.

The hemopoietic cell increase in the area subjected to temperature elevation concerned chiefly the granular leucocyte series, although there was a definite increase of hemoglobin carriers. A typical field showed stem cells, many eosinophile and heterophile myelocytes, a great many reticular and endothelial cells, and somewhat fewer erythrocyte precursors, polychromatic erythroblasts, and normoblasts. Megacaryocytes were frequently observed.

One of the deviations from the normal conditions in these tail loops was an interference of motion in the tail. It was at once observed that motion was as much restricted in the outside loop as it was in the intra-abdominal portions, so that the effect of immobilization on marrow was balanced and thus controlled in each animal.

Effect of Elevation of Temperature on the Intact Rat Tail in Anemia

None of the changes described in the preceding experiment approaches in amount the changes found in similar rats provided in addition with a stimulus to hemopoiesis as in anemia. The anemia was produced by splenectomy and by repeated bleedings.

Series 2.—Anemia through blood loss was produced by heart puncture under ether anesthesia every second day in 12 albino rats in which tail loops had previously been made as described above. It was possible to remove very large quantities of blood in this way, thus 47 cc. were bled from a 204 gm. rat in 23 days. The circulating hemoglobin was reduced to 25 to 40 per cent; the usual ground meal diet (purina fox chow) was supplemented by 1 per cent ferrous carbonate.

Splenectomy was done in another series of 16 rats, 8 of which had had fixed tail loops made. Confirming the observations of Lauda (30) the usual splenectomy on a tomized laboratory albino rat in this region (22) promptly develops an anemia as a result of infestation with *Bartonella*.

Several typical protocols are given.
Rat 9-40, 150 days, 200 gm.: Skin from distal half of tail denuded and tail sutured in peritoneal cavity, Mar. 27, 1935. Heart puncture every second day with removal of 31 cc. of blood between Apr. 1 and 15. caused hemoglobin decrease to 25 per cent. No further bleeding until the rat was killed on Apr. 3 when weight was 188 gm., hemoglobin 70 per cent. *Intra-abdominal portion* dense band of hemopoietic marrow at the metaphysis (Fig.

The epiphyseal spaces are filled with the same tissue and fat has completely receded from them. In the diaphysis there is still fat but this has been markedly reduced; the vessels are engorged and there are many islands of hemopoietic cells but fewer than in the solid bands at the epiphyses. *Outside loop*: almost completely fatty, there are a few scattered clumps of erythropoietic cells in the region of the epiphysis (Fig. 5).

Rat 9-29, 100 days, weight 112 gm., hemoglobin 15 gm.: Splenectomy and formation of fixed tail loop Apr. 24, 1935. Hemoglobin 5 gm. on May 3, Rat killed May 28, when hemoglobin was 9 gm. In the gross the marrow was yellow in the *outside loop* and microscopic examination showed fat with no signs of hemopoiesis. The diaphyseal regions of the *intra-abdominal portion* were also yellow, but there was a 2 mm. broad band of bright red hemopoietic tissue at each metaphysis. Histologically the bright band was seen to be composed of an uninterrupted sheet of hemopoietic cells. In the diaphysis, the fat cells were interspersed with numerous clusters of hemoblasts.

These experiments show very much more hemopoiesis in the portion of the tail which had been situated in the peritoneal cavity than in the outside loop. The effect of this operation on the formation of red marrow is enhanced by the anemia produced regardless of production by hemolysis or blood loss, and in all cases the gross and histological picture was unequivocal in demonstrating an increased medullary activity in the warmer bone marrow.

A further control series of 20 normal rats has been studied where anemia was produced by cardiac puncture, as much as 76 cc. of blood being removed in 47 days, and will be presented in a subsequent report. In 10 of these animals, red marrow formed in the tail in varying amounts and in a similar number no changes could be detected in the fatty marrow, showing that red marrow can form in the tail of old rats under certain conditions, the causes of variation in response being at present unknown. Similar variations in response to anemia were found in red marrow spread in the extremities of dogs (12) and rabbits (32). We wish to emphasize here the greater formation of bone marrow in the locus where the temperature had been elevated as compared with control areas of the tail.

Free Autogenous Transplantation of the Rat Tail (and Foot) to a Warmer Environment

At birth and for some 12 hours after, the tail consists of a series of vertebral elements interspaced with well developed notochordal remains; most of the vertebral mass at the poles consists of flattened

cells of undifferentiated mesenchyma; the equatorial region consists of precartilaginous, a short row of more swollen hypertrophic cells with small nuclei, but bone and cartilage are absent. At 12 to 24 hours the cartilage becomes swollen, hypertrophic, and calcifies. On the 3rd day the equatorial region consists of calcified cartilage which is infiltrated with hemopoietic bone marrow cells and surrounded at the periphery by bone; proceeding toward the poles there is found hypertrophic cartilage, small flat cartilage cells, and mesenchyma. The 6 day tail has its center filled with a dense accumulation of bone marrow, otherwise bone and cartilage have increased, mesenchyma has decreased. At 13 days the distal vertebrae have lost most of the red bone marrow cells, which have been replaced by fat. The red marrow then retreats centripetally almost to the sacrum. The adult condition in the distal caudal vertebrae, consisting of fat, macrophages, vessels and nerves is reached at 70 to 90 days after birth.

The idea behind the present experiment was that if a decrease of temperature in the outlying tail were responsible for the atrophy of the red marrow, then the tail when transplanted at an early stage to a region where the temperature was persistently warmer than its normal environment, should retain its hemopoietic marrow. Accordingly the tail was amputated as early as possible in neonatal life and transplanted to the peritoneal cavity as a free graft.

Series 3.—Free tail transplants had been made by Paul Bert (26) who amputated 2 mm. of a young rat's tail and transplanted it beneath the skin of another; 2 months later it was twice the original size. Marchand (27) observed growth in free autogenous subcutaneous transplants of the tail in 2 rats.

In the present experiments 19 rats were operated upon 3 to 6 hours after birth. It was helpful to wait until the rat had the stomach distended with milk for technical reasons, because when an incision was made in the peritoneal cavity it was exceedingly difficult to successfully replace the prolapsed viscera. The full stomach, however, may be seen through the abdominal wall; wherever it presented itself a 2 mm. incision was made, usually in the lateral abdominal wall; the tendency of the stomach to herniate obturated the hole so that small viscera could not protrude. The mesenchymal tail was amputated, skinned, grasped by its butt end and pushed inside the peritoneal cavity, the skin was coapted with the fingers and closed with two fine silk sutures.

Unless the skin was removed, the tail died and sebaceous cyst formation was found at autopsy.

The tail quickly became adherent to adjacent tissue; at times a mesentery was developed in which the delicate vessels supplying it were seen; in one instance the experiment failed due to lack of attachment of the graft which was found as a series of pearly calcified cartilaginous beads devoid of soft tissue. The rats were killed at first at 4 day intervals, later weekly and monthly.

The free grafts otherwise survived and went through in normal time relationships the functional changes up to the point of development of red marrow; growth occurred and the evidence is presented in x-rays (Fig. 6). An important difference was thereafter observed in that there was less infiltration of fat, and the red marrow persisted in large amounts for at least 1 year, far past the time when it has entirely receded in the normal tail position.

The significant data observed in the development in these free grafts may be summarized as follows:

4 day graft: Hemorrhage was seen in the connective tissue surrounding the vertebrae; areas of the cartilage showed impairment of nutrition by staining palely but most of the graft was alive and calcified cartilage was seen in the middle of the segment.

8 day graft: There was a size increase in the surrounding connective tissue. Small necrotic areas were seen, but occupied proportionately less of the graft than at 4 days; the necrosis occurred usually along one side of the graft. The notochordal remains appeared normal, but were distorted by the cartilage necrosis so that they did not occupy the midline. Bone marrow cells infiltrated the center of the calcified cartilage as in the normal 6 day tail. A slight amount of bone was seen.

15 day graft: This showed almost complete disappearance of the intervertebral disc fibers; in this and all subsequent sections there was resulting fusion of the cartilage masses of adjacent vertebrae which in later stages became replaced by bone. Extensive hemopoietic bone marrow was found partially infiltrated with fat; the cellular bone marrow consisted of nucleated red cells, megacaryocytes, and all other hemoblast precursors. The bone was extensively developed, trabeculae had appeared.

24 day graft: In addition to the findings at 15 days no necrotic elements were seen. Development of the epiphyses had occurred and these contained slight cellular marrow. There were, however, much more extensive deposits of hemopoietic marrow in the main vertebral body, most extensive at the metaphyses, but also in the shaft where they were somewhat diluted by fat infiltration.

60 day graft: The vertebrae were united by bone where there had been carti-

luginous fusion. There was greater infiltration of fat than at 24 days. Much hemopoietic tissue was still present, although there was some fat in the diaphyses.

100 day graft: There has been a great increase in growth in the tail vertebrae. Dense hemopoietic aggregates fill the epiphyseal regions and a part of the diaphysis. There has been a slight increase in the amount of fat present in the marrow, mostly in the diaphysis as compared with the 60 day specimen, but the fat content is much less than in a normal adult rat tail.

205, 256, 311, 359 day grafts: These specimens resemble each other and the 100 day graft. Bone, cartilage and notochordal remains persist with living cells. The metaphyses are filled with hemopoietic tissue chiefly of the granulocytic series but with many red cell forms and megacaryocytes (Fig. 7). The epiphyseal marrow spaces contain a slight amount of fat but much cellular marrow. The diaphysis contains mostly fat with some red marrow cell clusters.

In this experiment then, the tail was amputated at a period before bone or bone marrow had developed and transplanted to the peritoneal cavity where the temperature greatly exceeded the temperature of the tail. The graft survived and developed essentially normally, aside from an initial minor necrosis. The bone marrow maintained in very large part its red marrow in the epiphyseal and metaphyseal regions where it would have undergone complete fatty substitution in the normal location. Fatty marrow developed in the middle of the diaphysis just as it would have in the normal position. There was thus found a fundamental difference in the development and retention of hemopoietic marrow of the tail which developed in the peritoneal cavity as compared with the marrow of the tail developed in its normal site.

The circulation in the rat tail at birth is very slight and this structure could be amputated with loss of only a tiny drop of blood. The circulation of the grafts was obtained from any neighboring structure and the avascular cartilage developed a complex sinusoidal circulation, showing that the capacity to form sinusoids is determined by the cartilaginous mass rather than the ingrowing endothelium. In other words, the character of the tissue determines whether the ingrowing endothelium is sinusoidal or not.

Since the foot is an outlying region in which hemopoiesis yields to a fatty marrow during development, as a variant to the experiment just reported, in 18 rats at birth one foot was amputated, skinned, and inserted in the peritoneal cavity. An adequate control area was provided in the unamputated foot. In the transplant usually the phalanges disappeared but tarsus and metatarsus survived.

After 60 days, a great difference was observed in the hemopoietic bone marrow of the graft (Fig. 8) as compared with the fatty marrow of the normally developed foot (Fig. 9).

Free Transplantation of the Rat Tail to Other Rats in Warm and Cooler Loci

Series 4.—In this experiment 16 litters of rats were used within 6 hours after birth, when the tail of each rat was amputated, denuded of skin, and transplanted to either the peritoneal cavity or the dorsum of the foot of an adult rat under ether anesthesia. Both the mother and another rat were used in each case. It must be emphasized that the stock had been greatly inbred in this rat colony. The histological results in 35 grafts are reported, 20 to 150 days after transplantation.

The results may be divided into three groups. Nine grafts, all placed in the peritoneal cavity of the mothers, developed exactly like autogenous grafts in this location; they formed well organized vertebrae with cartilage, bone, and hemopoietic bone marrow which was retained in the epiphyses and metaphyseal regions as long as the experiment lasted. The red marrow (Fig. 10) consisted of erythroid and granulocytic elements with many megacaryocytes. The diaphysis was filled with fat for about one-half of its extent. Those grafts where red marrow formed were characterized by good anatomical organization. Nine grafts, all implanted in the feet of mothers developed equally well and contained completely fatty marrow (Fig. 11) after 5 months when the abdominal grafts of litter mate origin all had red marrow.

Five grafts with equally good cartilage and bone developed a decidedly different marrow from these consisting of a diffuse protein framework with many similar nonphagocytic mononuclear basophilic cells without differentiation into erythroid or granulocyte cells. There was a slight fatty infiltration in this marrow.

Twelve grafts resembled each other and were quite different from the groups just described in that cartilage and calcified cartilage formed with slight or no bone formation. The anatomical organization was poor and epiphyseal cartilage separated by rather large masses of fibroblasts was seen where bone marrow is normally present. While some of the cartilage and calcified cartilage was dead (absence of cells) much of the graft survived (Fig. 12). In these areas there were seen clusters of a very primitive myelocytic type of marrow with macrophages, but without erythroid elements.

In one-half of the grafts not developing this bizarre non-bony tissue, or primitive marrow, the differences in content of red and yellow marrow were related to abdominal and foot position and were exactly predictable on a thermal basis. The causes of the difference in re-action between grafts forming organized chambers and grafts forming disorganized cartilage remnants without bone could not be predicted. The disorganized state was seen when grafts were made to the mother as well as to some other animal, and it occurred in the abdomen as often as in the foot. Moreover it occurred in some of the tails of members of a litter grafted in a given host, while those of other litter mates in the same host formed normal appearing units. It is clear that cartilage was successfully grafted and grew in every instance; the defect seems to be in subsequent development and may be related to tissue groups somewhat analogous to the Landsteiner blood group effect, since red marrow makes erythrocytes.

Influence of a High Environmental Temperature on Bone Marrow

In order to exclude all operative procedures, 40 albino rats were kept at an elevated environmental temperature ($33-36^{\circ}\text{C}.$) for 21 to 70 days. It was impossible to keep rats at $38^{\circ}\text{C}.$, since all died in 2 to 4 days with lung infections. Bone marrow of feet and tail were studied at the conclusion of the experiment. Control rats for each age and sex were kept.

Method.—The rats were placed in an incubator, consisting of a wood box $4 \times 2 \times 2$ feet in dimensions, equipped with an electric heating coil built in the roof and insulated with asbestos. Temperature control was obtained by an aneroid thermostat. The box had glass windows on three sides, and electric lighting was uninterruptedly maintained. Temperature was measured by a thermo-electric couple and a mercury thermometer. For ventilation one of the glass windows was replaced by a perforated fiber board. The temperature range was $34.2-1.8^{\circ}\text{C}.$ The air was agitated continuously with an induction motor fan. No attempt was made to control humidity, which was probably low; the drinking water, always adequate in amount and changed twice daily, was kept in large inverted stoppered bottles furnished with a glass tube as outlet. The box was kept in a very small room without windows or heat, used for no other purpose. Food consisted of a mixture of dried meat, milk, and grain with cod liver oil (purina fox chow). Hemoglobin determinations were made on blood removed by heart puncture at the beginning and end of the experiment.

Series 5.—Typical protocols of three groups with controls are furnished in

Table I. All of the older rats lost weight while in the incubator, while young rats placed in the box after weaning, gained weight, but at a slightly slower rate than litter mate controls. There was marked polydipsia.

In many rats the testes showed profound atrophy involving all of the spermatogenic series of cells with giant cell formation in the testis tubules. In approximately one-third of the rats, either slight cytolysis or no change was detected in the testes.

TABLE I
*The Effect of Elevated Temperature on Bone Marrow of Feet, Tail, and on Testis of Rats Kept in an Incubator**

No.	Sex	Age	Time in incubator	Environmental temperature variation	Histological estimate of hemopoiesis	Carbon deposition in tail marrow	Carbon deposition in foot marrow	Histological estimate of testis atrophy	Weight change	Remarks
		days	days	°C.					gm.	
1	♂	163	33	32.8-35.5	++	+++	++	No atrophy	-6	Control for 1, 2 and 3
2	♂	163	33	32.8-35.5	+	++	++	Partial	-34	
3	♂	163	33	32.8-35.5	++	+++	+	Partial	-20	
4	♂	163	—	16-26	None	None	None	None	+24	
5	♂	270	24	33-36	Trace	++	++	Profound	-30	Control for 5 to 9
6	♂	270	24	33-36	Trace	Trace	+	Profound	-56	
7	♂	270	24	33-36	+	+	+	Profound	-46	
8	♂	270	24	33-36	Trace	+	+	Profound	-45	
9	♂	270	24	33-36	+++	+++	+++	Partial	+18	
10	♂	270	—	16-26	None	None	None	None	-126	Control for 11 to 13
11	♂	>365	23	33-36	++	+++	++++	Profound	-158	
12	♂	>365	22	33-36	+	++	++	—	-77	
13	♀	>365	23	33-36	++	++	++++	—	+18	
14	♀	>365	—	18-26	None	None	None	None	—	Ovariectomized

* + + + + indicates maximal hemopoietic response.

The bone marrow of the feet showed hyperplasia in all of the animals. The marrow of the tail showed slight or moderate hyperplasia in each case but in no case did it approach the large amounts seen in operations where marrow was maintained at peritoneal temperature. The changes consisted of formation of clumps of cells at the metaphysis, mostly of the myelocytic and macrophage series; slight erythropoiesis and megacaryocyte formation was observed. In young animals inserted in the box after weaning, infiltration of fat occurred in the

diaphysis as in the previous experiment; the metaphyseal marrow cells were well preserved.

DISCUSSION

The evidence presented has dealt with the formation and maintenance of hemopoiesis in bone marrow normally yellow after the developmental period. The experimental procedures were designed to and indeed did produce an elevation of temperature of yellow marrow chambers. An effect occurred in each case resulting in blood cell formation in fatty marrow. We feel therefore that the results support the hypothesis that temperature variations of the order present in the extremities of normal mammals and birds (29) significantly affect marrow activity.

In addition to the experiments several supporting facts can be adduced which derive from previous findings. The observation that all bone marrow in newborn mammals is red supports the suggestion that the abdominal warmth is a factor in determining marrow distribution, especially since red marrow regresses soon after birth in cooler skeletal areas. Knipping (34) found in 2 dogs that recovery from the hemolytic anemia caused by pyrodine was more rapid in tropical than in temperate climates. Barcroft and coworkers (36) found an increase of reticulated red cells in the circulating blood of two men kept for several days in a glass chamber at 32–35°C. The observations of Sasybin (37) on animals and man exposed to very high temperatures are not comparable with the present experiments; red marrow spread occurred in all of the bones during the period of disturbed compensation to excessive heat, and an anemia was present.

In general there are two ways in which a physiological elevation of temperature may affect the bone marrow, namely a primary effect on tissue metabolism and a secondary vasomotor effect; while both effects are presumably operative, no evidence could be derived from these experiments as to the mechanism by which an elevation of temperature facilitated hemopoiesis.

In the first place, it is chemically reasonable to suppose that tissue fabricating large molecular compounds like hemoglobin, complex nucleic acids, and antibacterial agents would be greatly affected by fluctuations of temperature of the order of 6–10°C., as occur in normal

one marrow (29). Changes in temperature in an organism are in general very important: "on thermo-dynamical grounds this fact is not difficult to understand and its biological implications are manifest" (38). In blood itself, with variations of temperature there are appreciable changes in the masses of the components as well as in the solubilities of gases and in chemical affinities; there occurs on lowering of temperature a marked decrease of dissociation of oxyhemoglobin (23) as well as changes in carbonic acid-bicarbonate concentrations and pH (39, 40). The van't Hoff (43) law concerning the increased velocity of chemical reactions with temperature increase apparently applies to general metabolism in man with increased temperatures (35). Tipton (33) found an increased metabolism of nucleated vertebrate red cells with temperature increases.

Other complex structures are known to be affected by thermal differences of this order. Moore (24, 25) found that the testis is adversely affected by thermal increase to the body-trunk level; the spermatogenic cells of the testis atrophy or fail to develop when this structure is exposed to a climate as warm as the peritoneal cavity, as was also found in our experiments at a slightly lower thermal level. Schultz (41, 42) found that the black winter pigmentation of the body tips in the Russian rabbit could be produced by experimental application of cold.

It is common knowledge that vascular tonus responds to increase of temperature by relaxation and vasodilatation. In the warm environments in the present experiments, red marrow formed in the free grafts in connection with a newly formed circulation, and in the other experiments it formed or was preserved with the original circulation of the marrow intact. It was found that red marrow was located chiefly in the epiphyseal and metaphyseal regions, undoubtedly because of circulatory effects since the circulation of tail marrow (Fig. 13) shows vastly greater capillary accumulations here than in the diaphysis, even when the whole chamber contained only fatty marrow. The reason for this vascular accumulation in these regions needs further analysis. The retention of red marrow longest in the epiphyseal regions of marrow chambers where capillaries are most concentrated confirms the widespread view of many earlier writers, but it must be emphasized that the normal distribution of red marrow is not purely a question of

epiphysis *versus* diaphysis; for example, while the diaphysis contains red marrow the lowest epiphysis of the mature dog femur usually contains yellow marrow. Epiphyseal predilection accounts for the fact that red marrow often is held longer in these regions than in the shaft, but it in turn yields to segmental regression as the extremity matures.

It is our conception, based on the experimental evidence, that there are two types of fat infiltration in marrow. The first type present in outlying regions of the skeleton, is chiefly a local topographic effect, and was overcome in these experiments presumably due to the elevation of temperature. The second type is generalized and involves the central bones in healthy adults, and is probably related to advantageous or disadvantageous local sinusoidal effects (Fig. 13), the physiology of which is unknown. This is the effect by which fatty infiltration in the diaphysis of free tail grafts in warm loci is produced, as well as the epiphyseal location of hemopoiesis in tail loop experiments. This is the effect on which the transitory retention of red marrow in the epiphyses as well as fat infiltration in deep bones may be explained. The epiphyseal predilection accordingly is explainable on the basis of a more favorable vascular effect in this region than in the diaphysis. The fatty infiltration in normal bones of the body trunk is due to a less favorable vascular effect than in the non-fatty regions. The second or generalized type of fatty infiltration depends to some extent on the state of nutrition of the animal, especially with respect to the *Bausteine* of blood (32, 47), since in starvation fatty marrow is replaced by a gelatinous non-fatty condition. It also depends on the oxygen tension of the blood, since bone marrow fat decreases in anemia and on mountains. It is possible on an experimental basis to produce differences as demanded by this theory. (a) In local tail loop, incubator, and free abdominal grafts, red marrow is produced or maintained in epiphyseal regions but not in diaphysis, since the generalized vascular mechanism is not favorable. (b) In anemia in many cases (12, 44, 45) and under diminished oxygen tension as at high altitude (46), red marrow spread does not uniformly involve all outlying bones, since local conditions (thermal decrement) interfere. (c) In the present experiments in anemia in animals where a portion of the outlying marrow is at an elevated temperature, all of the warm outlying bone is involved in marrow hyperplasia.

Referring again to the testis as affected by heat, it may be seen that the bone marrow presents a converse situation to this gland. Under physiological conditions a *maximal* temperature exists for the testis and increase beyond this abolishes gametogenesis, whereas in the bone marrow a *minimal* temperature affects hemopoiesis adversely.

The difference in response to homogenous grafting of rat tails to other rats deserves a word of comment. Cartilage apparently was always successfully grafted to non-donors, but in many animals further differentiation into bone is absent, and poorly organized cartilaginous remains with an exceedingly primitive marrow result. If this effect is due to tissue cell groups, obviously the defect comes in differentiation rather than in cartilage growth.

SUMMARY

A great difference exists in the adult bone marrow of central bones as compared with outlying bones of the mammalia and avia, the distal bones being at a great disadvantage from the standpoint of blood cell production. Several experimental procedures are reported by which this disadvantage is overcome and in consequence fatty marrow of outlying bones is replaced by red marrow occurring chiefly at the epiphyseal regions, unless a low oxygen stimulus is also provided when marrow of the diaphysis becomes involved. A common factor in all of the experiments was an elevation of temperature beyond that prevailing in these distal regions, and it is felt that the evidence warrants the opinion that the cause of improvement is thermal. In some experiments, blood cell formation was increasing while the heat was adversely affecting the testis. The experiments permit construction of a general theory of fat distribution in bone marrow.

In certain grafts of precartilage to other rats, normal differentiation into bone, cartilage, and marrow occurred, while in others cartilage and very small amounts of primitive marrow developed with slight, or no bone formation. Cartilage was always successfully engrafted.

The capacity to form sinusoids in bone marrow is determined by the nature of the tissue rather than by the ingrowing endothelium.

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EXPLANATION OF PLATES

Photographs of sections stained with hematoxylin-eosin. Except in Figs. 8 and 9, a portion of epiphyseal cartilage (C) is shown to provide comparable loci. All specimens were decalcified with 5 per cent HCl, except Figs. 6 and 12.

PLATE 13

FIG. 1. Rat B, series 1, tail loop operation: Portion of the tail inserted in peritoneal cavity for 86 days. $\times 220$.

FIG. 2. Rat B, series 1, tail loop operation: Outside loop region of the tail of same rat as Fig. 1, a completely fatty marrow. $\times 220$.

FIG. 3. Rat 8-85, series 1, tail loop operation: Portion of the tail inserted in peritoneal cavity for 38 days, showing many dilated capillaries and moderate hemopoiesis. $\times 220$.

FIG. 4. Rat 9-40, series 2, tail loop operation in anemia: Portion of the tail inserted in peritoneal cavity for 34 days; 31 cc. of blood removed by heart puncture in first 14 days after operation. $\times 310$.

PLATE 14

FIG. 5. Rat 9-40, series 2, tail loop operation in anemia: The outside loop region of the same rat as Fig. 4 (short loop). $\times 390$.

FIG. 6. X-rays of end-results in series 3, following free transplantation of tail to peritoneal cavity with a control specimen ($\frac{1}{2}$ day). The x-rays shown were made at the same focal tube distance of specimens removed 9 to 100 days after grafting; no calcification was present in the control tissue and the development and growth of the bony units may be seen.

FIG. 7. Series 3, autogenous free transplantation of tail to peritoneal cavity after 359 days. This rat had been injected with India ink 4 and 2 days before necropsy, and carbon granules can be seen in some of the macrophages. $\times 640$.

PLATE 15

FIG. 8. Series 3, autogenous free transplantation, 4 hours after birth, of foot to peritoneal cavity after 167 days, showing dense marrow accumulation in heel bone. $\times 110$.

FIG. 9. Series 3, showing marrow of heel bone, of the control unamputated foot of the same rat as in Fig. 8. $\times 110$.

FIG. 10. Series 4, homogenous transplantation of tail at birth to the peritoneal cavity of mother, after 182 days. $\times 110$.

PLATE 16

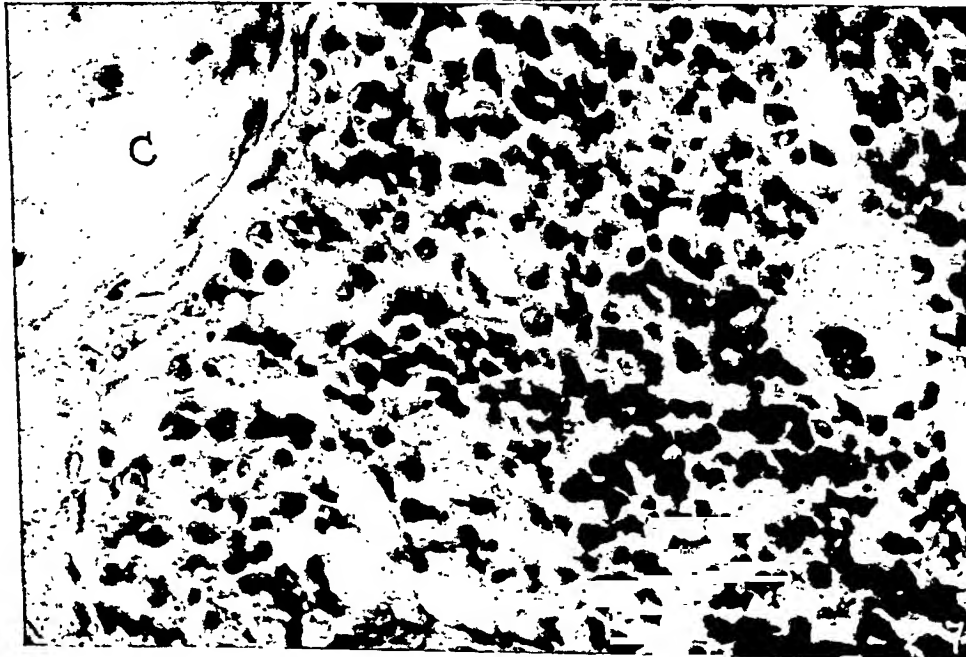
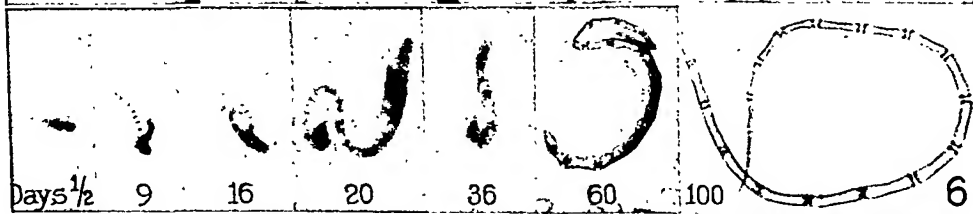
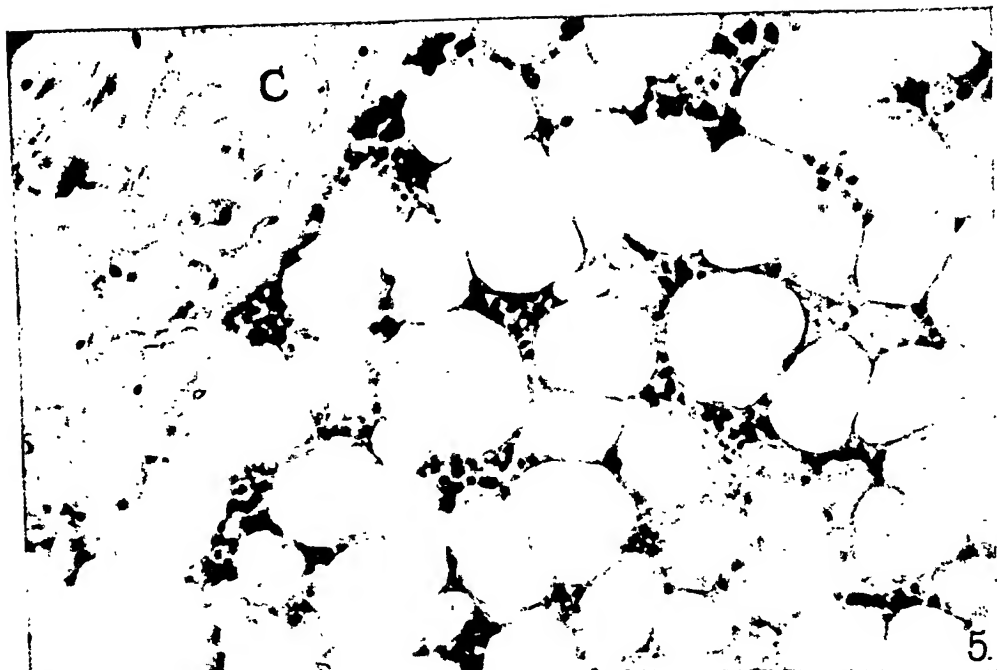
FIG. 11. Series 4, homogenous transplantation of tail at birth to foot of mother after 182 days: The tail was transplanted from a sibling of that illustrated by Fig. 10, to the same recipient. $\times 690$.

FIG. 12. Series 4, homogenous transplantation of tail at birth to abdomen of mother after 150 days: This shows the atypical formation of cartilage (C), calcified cartilage (CC), and dead calcified cartilage (DCC). No traces of bone were found.

FIG. 13. Cleared specimen showing circulation of tail in an old rat. This rat was injected with 17 cc. of 20 per cent ink during the period until death occurred, 5 minutes after beginning the injection. Although the marrow of these vertebrae was yellow, much greater capillary accumulations of ink are evident in the metaphysis and epiphysis than in the diaphyseal region.

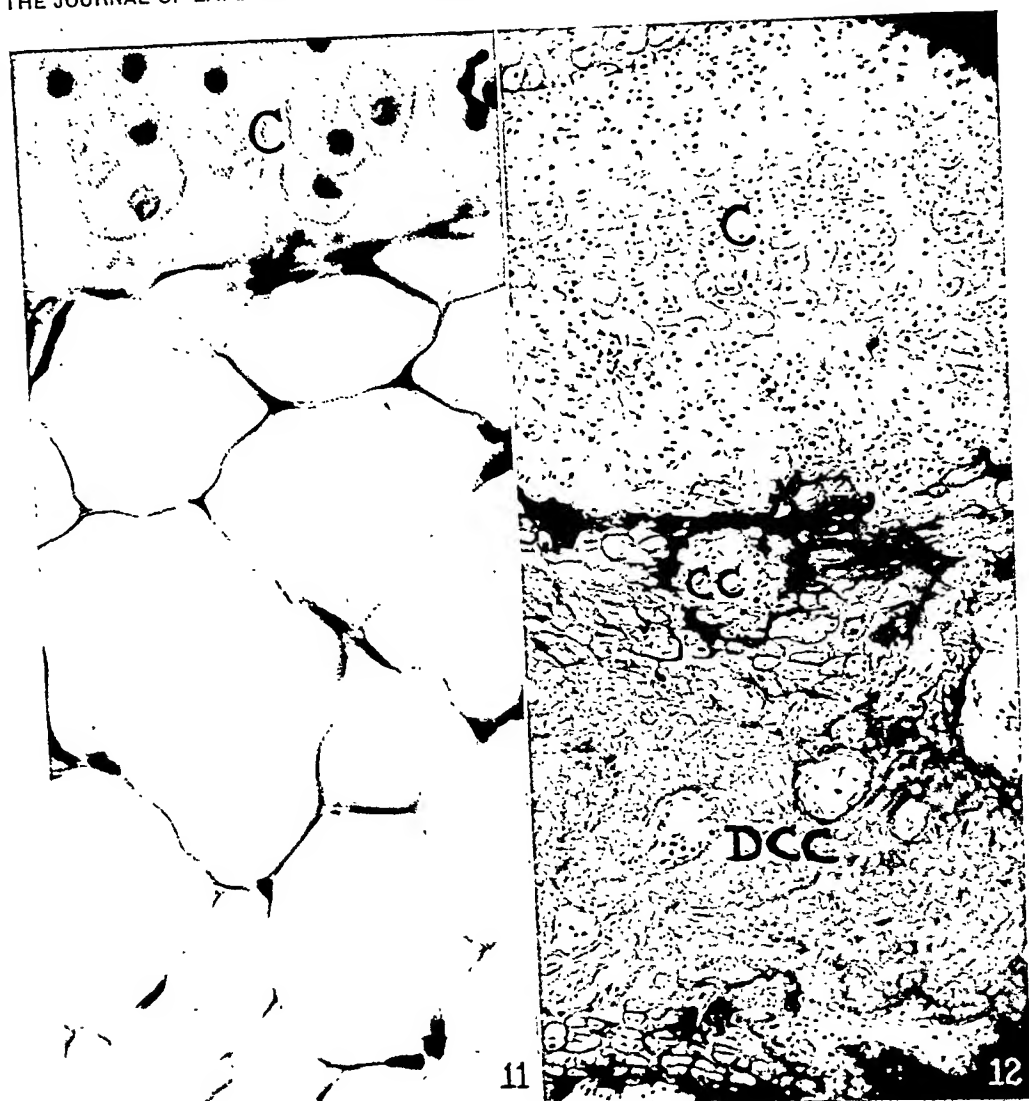


(Huggins and Blocksom: Temperature increase affecting bone marrow)





(Huggins and Blocksom: Temperature increase affecting bone marrow)



13

AN INCREASE IN RETICULO-ENDOTHELIAL CELLS IN OUTLYING BONE MARROW CONSEQUENT UPON A LOCAL INCREASE IN TEMPERATURE*

BY CHARLES HUGGINS, M.D., AND W. J. NOONAN, M.D.

(From the Department of Surgery, The University of Chicago, Chicago)

PLATES 17 TO 19

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Methods by which an increase of hemopoiesis in the rat can be induced in outlying bones are described in an accompanying paper (1). Concomitant with an increase of red marrow in the experiments just recorded, there was always found an increased number of cells of the reticulo-endothelial system in bone marrow and the present communication deals with this increase in a parallel series of experiments. Wislocki (9) found in normal bone marrow that the distribution of carbon particles in the cytoplasm of reticulum cells and in the endothelium lining the marrow capillaries coincided closely with the normal distribution of hemopoietic tissue. In our work these findings were confirmed in general and extended to increases of hemopoietic tissue.

In the bibliography (2-10) is traced the development of some of the present concepts of the series of cells in bone marrow with the peculiar capability of ingesting finely divided particulate matter. Stress may be laid on two important researches. Ribbert (5) first drew attention to the fact that certain endothelium such as the lining of the liver sinusoids is quite different physiologically from the lining of capillaries supplying most of the structures of the organism, in that sinusoidal endothelium is capable of ingesting particulate matter, a property not shared with the capillaries supplying muscles, brain, and many other structures. A new technique invented by Rous and Beard (11, 12)

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has greatly broadened the approach to study of the reticulo-endothelial system. These investigators successfully cultured liver clasmatoctes *in vitro* and found that these cells were very large with an immense circular membrane, slowly motile, and extraordinarily sticky. A morphological difference between liver and spleen clasmatoctes was observed. No studies on bone marrow macrophages with this technique have so far been reported.

The bone marrow fraction of the reticulo-endothelial system will be described in several species of normal animals and in five series of experiments parallel with those reported in an accompanying paper (1).

Series 1.—Construction of tail loops, in which the distal portion of the tail was maintained in the abdominal cavity by surgical anastomosis.

Series 2.—The same as series 1 in rats anemic from blood loss and *Bartonella* infection (splenectomy).

Series 3.—Free transplant at birth of tail or foot to peritoneal cavity of the donor rat.

Series 4.—Free transplant at birth of tail to abdomen and foot of mature rats.

Series 5.—Maintenance of rats in a warm box at a temperature of $34^{\circ} \pm 1.6^{\circ}\text{C.}$ for 21 to 33 days.

Methods

Intravenous injections of Higgins' American India ink freshly prepared in 12.5 per cent concentration in 0.9 per cent sodium chloride aqueous solutions were made in two sittings and the animals were sacrificed 2 or 3 days after the final injection. In the rat, the dose used for each injection was 1 cc. for each 80 gm. of body weight, while in rabbit, cat, monkey, and fowl weighing 3 kilos, injections of 20 cc. on each of 3 successive days were well tolerated. All injections were made in leg veins, and in rats under ether anesthesia.

The rats of the series in the warm box frequently died when replaced in the incubator after ink injections, so that in series 5 the animals were kept at room temperature after the injections were started.

The material was studied by histological methods, using paraffin technique, and by making the carcass transparent by the Spalteholz method (13, 9), except that synthetic methyl salicylate was used as a clearing agent instead of natural wintergreen oil and benzyl benzoate was found not necessary. Some of the cleared specimens developed an interfering brown color in the muscles instead of the more desirable light yellow tinge and it was found that this could be avoided by fixation and subsequent treatment of each specimen in a separate container.

Air entrapped in the tissue was removed by suction in an evacuated jar when clearing was complete.

RESULTS

Histological Findings.—

Microscopic examination of the histological sections of bone marrow prepared in this way showed carbon accumulations almost exclusively in cells of the reticulum and the endothelial lining of the sinusoids. Occasionally carbon particles were found in monocytes and polymorphonuclear leucocytes; a rare extracellular mass of carbon could be satisfactorily explained as a technical artifact.

In normal yellow bone marrow in outlying bones where no trace of blood cell formation could be detected, there were found granular carbon deposits in reticulum and sinusoidal endothelium. These cells were found sparsely scattered but present in all yellow bone marrow. Otherwise our findings confirmed the observation of Wislocki (9) regarding the distribution of these specific phagocytes in relation to hemopoietic foci. Wherever hemopoiesis was taking place the macrophages were greatly increased in number as compared with inactive yellow bone marrow.

Translucent Specimens.—

Normal Animals.—A difference was at once apparent in all mature mammals studied in the amount of carbon taken up by the bone marrow of central bones of the body as compared with the outlying bones of the extremities (limbs and tail). These distal bones of normal animals contained little carbon and were transparent; the marrow of central bones on the contrary was jet black.

Rat: At 16 days, all of the bones contained much carbon (Fig. 1); even at this age the relative accumulation in the metaphyseal region of the tail is seen. At 27 and 43 days (Fig. 2) retention in tail metaphyses and feet is still visible. In adult rats all of the body vertebrae were jet black as far as the segment distal to the last vertebra equipped with a lateral bony process; the tail beyond this point was translucent (Fig. 3). Dense carbon masses were seen in all of the bones of the adult rat except the tail, hands, and feet, the carbon deposition rather abruptly ceasing at ankle and wrist joints with occasionally a few fine deposits in foot bones. *Cat:* In half grown cats, carbon accumulation occurred in the metaphyseal regions in the distal portion of the tail. In 4 mature cats the proximal half of the tail contained solid black carbon masses in the proximal 8 or 9 tail vertebrae while the distal tail was translucent, and in 2 adult cats only the first inch of the

tail beyond the sacrum contained carbon visible in the gross. *Dog*: In a young animal weighing 5400 gm. the proximal half of the tail was dense with carbon accumulations, and the distal half had disc-like metaphyseal collections. In 2 mature dogs weighing 20 and 25 kilos no carbon was visible in the gross in the tail. In 6 months old *pigeon*, *hen*, *canary*, and *guinea pig* (Fig. 10) carbon accumulation, dense in femur and upper tibia, stopped abruptly at junction of middle and distal thirds of the tibia and distal to this point all of the bones were transparent. In mature *Macacus rhesus* and *rabbit* carbon deposition extended in femur and tibia to ankle joint and the foot bones were perfectly clear; in the macaque, two tail vertebrae distal to the sacrum were solidly black with carbon, the two next distal vertebrae had accumulation only at the metaphyseal regions, and beyond this the tail was transparent, the transition point of carbon with non-carbon marrow being very sharp.

Experimental Series.—The details of the experimental procedures are given in an accompanying paper (1). Typical results are shown in Figs. 3 to 9. Attention is drawn to the indicators of increased reticulo-endothelial activity, namely foot and tail marrows. Perhaps the only comment necessary concerns series 1, in which tail loops were established where differences occurred in the marrow of the *outside loop* depending on whether it were long and projected far from the body (Fig. 4) or short and closely applied to the trunk (Fig. 5). When short, the outside loop contained far more reticulo-endothelial and hemopoietic tissue than when the loop did not come in contact with the body. In loops closely applied to the warmer trunk, there were increased thermal effects.

DISCUSSION

The present results are at variance in two details with the findings of Wislocki (9). This worker stated that in the dog and cat carbon was not visible in the marrow in the gross, whereas in our experiments the marrow of these species did not differ from that of other mammals; the discrepancy can probably be harmonized by differences in dosage in the two investigations. Wislocki also found that carbon was abundantly present in all of the vertebrae, including those of the t of the adult rabbit, while in 6 of these animals examined by us there was found a great difference between the vertebrae of the b including the proximal half of the tail, which were jet black in and those of the distal half of the tail which were transparent.

SUMMARY

In adult mammals and birds there is a great quantitative difference in the reticulo-endothelial system content of the bone marrow of central bones as compared with distal outlying bones.

Experimental procedures reported in the accompanying communication effecting development and increase of hemopoiesis in inactive yellow marrow also effect a reticulo-endothelial cell increase.

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EXPLANATION OF PLATES

These photographs were made from specimens injected with colloidal carbon and cleared with methyl salicylate after dehydration with alcohol. Unless otherwise specified, the animals were injected with a 12.5 per cent solution of India ink, 4 and 2 days before death, and each dose was at the rate of 1 cc. for each 80 gm.

PLATE 17

- FIG. 1. Rat, age 16 days. Dose: 0.75 cc., 6 hours before death.
- FIG. 2. Rat, age 43 days. The opaque masses in the upper abdomen are carbon depositions in liver and spleen.
- FIG. 3. Rat, age 156 days. Litter mate control for Fig. 4.

PLATE 18

- FIG. 4. Rat, age 156 days. Series 1: Long outside loop constructed at operation at 23 days. The circular black silk thread denotes point of anastomosis with peritoneal cavity, and tail distal to this point had been inserted in abdomen.
- FIG. 5. Rat, age 87 days. Series 1: Short outside loop constructed at opera-

tion at age 27 days. The greater density of the intra-abdominal tail as compared with the external loop can be seen.

FIG. 6. Rat, age 205 days. Series 3: Autogenous transplant of tail to peritoneal cavity made 3 hours after birth. The dense black marrow of the tail transplant is seen in the left quadrant of the upper abdomen (arrow). Note the transition from black to translucent marrow in the amputation stump of the tail below the pelvis.

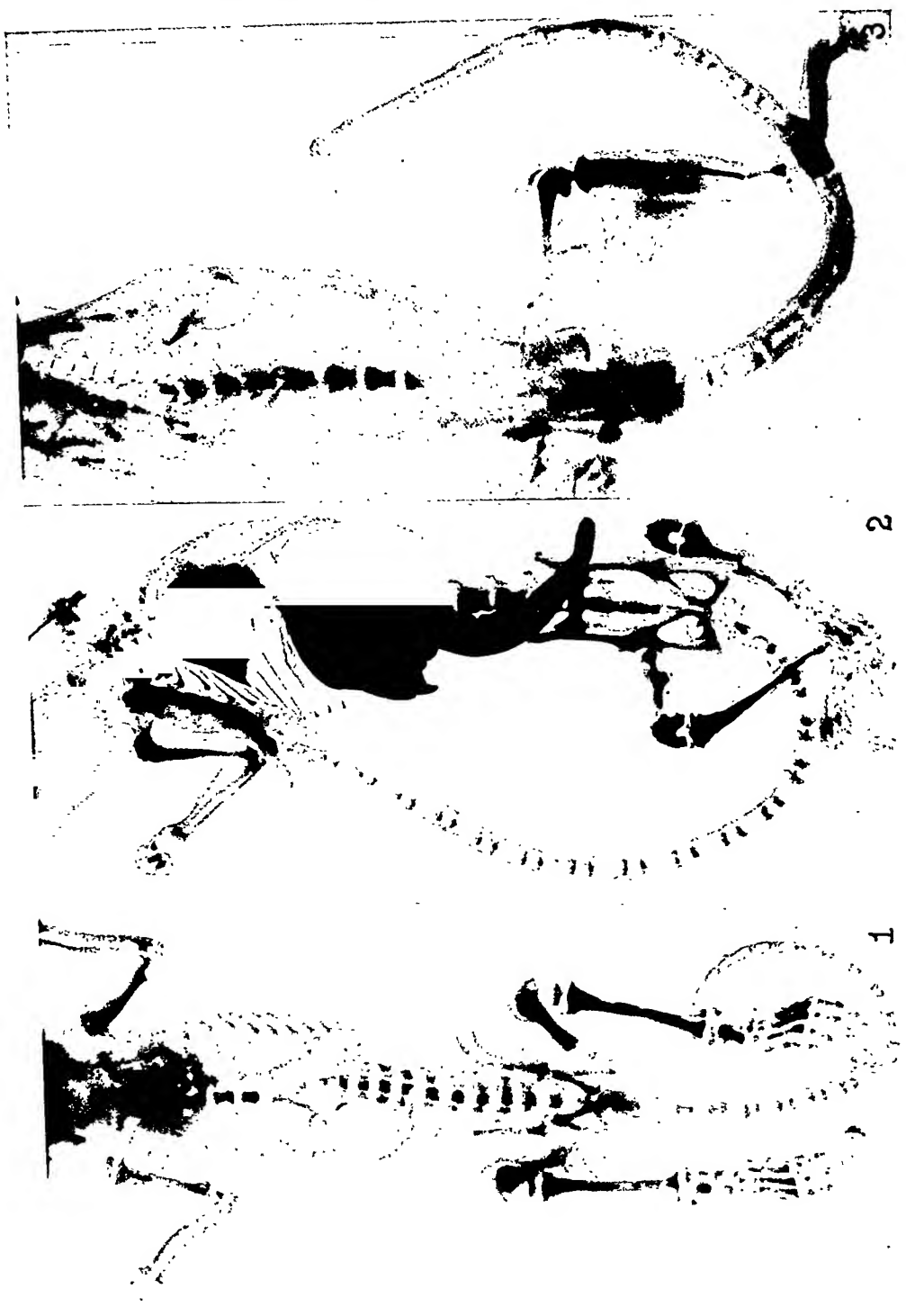
PLATE 19

FIG. 7. Rat, age 228 days. Series 3: Autogenous transplant of foot to left upper peritoneal cavity made 3 hours after birth. The blackness of the transplant (arrow) may be compared with the normal unamputated foot which serves as a control area.

FIG. 8. Rat, age 230 days. Series 4: This rat was the mother of a litter of 7 rats, the tails of which were amputated, skinned, and transplanted to the mother 18 hours after birth. Two tails were transplanted to the peritoneal cavity and abdominal wall respectively, and one tail in each of the mother's feet. The 6 grafts can be seen (arrows). Compare deposition of carbon in the abdominal grafts with the translucent foot specimens.

FIG. 9. Rat, age more than 1 year. Series 5: This rat was kept in a warm box at a temperature of 33–36°C. for 31 days.

FIG. 10. Guinea pig, normal adult, weight 650 gm. The sharp line of termination of carbon deposition in the tibiae can be noted and the jet blackness of the central bones compared with the outlying bones of the feet.



(Huggins and Noonan: Temperature increase affecting bone marrow)



(Huggins and Noonan: Temperature increase affecting bone marrow)



7



8



9



10

(Huggins and Noonan: Temperature increase affecting bone marrow)

STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

II. CERTAIN DISTINGUISHING PROPERTIES OF TWO STRAINS OF PNEUMOCOCCUS TYPE III VARYING IN THEIR VIRULENCE FOR RABBITS, AND THE REAPPEARANCE OF THESE PROPERTIES FOLLOWING R→S RECONVERSION OF THEIR RESPECTIVE ROUGH DERIVATIVES

BY MORRIS F. SHAFFER,* PH.D., JOHN F. ENDERS, PH.D., AND
CHAO-JEN WU,* M.D.

*(From the Department of Bacteriology and Immunology, The Harvard Medical
School, Boston)*

PLATE 20

(Received for publication, May 11, 1936)

In the foregoing paper (1) it has been shown that while strains of Pneumococcus Type III incapable of multiplication at 41°C. are avirulent for the rabbit, not all strains able to persist and to grow at this temperature are equally lethal for that animal. Two smooth strains of the latter group exhibiting a striking disparity in their rabbit virulence have been studied more closely, and evidence obtained which indicates that the variation in virulence among such strains may depend at least partly on differences in size of the capsule and the rate at which this structure is lost.

The findings suggested an inquiry as to whether the capsular characteristics depend on factors which can be varied in the manner in which it has been demonstrated by Griffith (2), Dawson (3) and others that type specificity can be altered, or whether they might be the result of more stable physiological processes, which, although temporarily inapparent during existence in the R phase would again reappear unchanged on the resumption of the S form, regardless of whether the

* At the time this work was done Dr. Wu held a Travelling Fellowship from the Peiping Union Medical School, and Dr. Shaffer a Fellowship in the Medical Sciences from the National Research Council.

strain SV, 0.5 cc. quantities of the antisera used were twice absorbed with the deposit from 100 cc. of 0.1 per cent dextrose infusion broth culture, allowing a period of exposure to each absorbing dose of 1 hour at 37°C. 0.5 cc. of the antisera employed in demonstrating the similarity of old cultures of CH and SV to R variants in their agglutinative behavior were absorbed twice with the deposits from 20 cc. of 5 and 10 hour dextrose serum broth cultures of CH and the R variant respectively.

Capsule Stain.—After trial of many of the capsule stains in general use, none of which were entirely satisfactory, the following stain was devised. Organisms either from culture or the animal body are emulsified on the slide in a drop of normal rabbit serum. The air-dried unfixed preparation is then stained for 1 minute with an alcoholic solution of methyl violet (to 1 part saturated solution of methyl violet in ethyl alcohol filtered through paper is added 1 part of ethyl alcohol). The excess stain is shaken off and the slide flooded with an alcoholic solution of basic fuchsin (to 1 part saturated solution of basic fuchsin in ethyl alcohol filtered through paper is added 4 parts of alcohol). This stain is allowed to act for 20 seconds and not longer. The preparation is then washed very rapidly in water and immediately blotted dry.

Method Used in Determining Relative Size of Organisms.—Calibrated Hopkins centrifuge tubes were employed, in a method similar to that of Jones and Little (6) in their study on increase in size of bacteria after sensitization with antiserum. Using the same cleaned pipette for all determinations, equal volumes (usually 0.9 cc.) of the various cultures were placed in the Hopkins tubes and centrifuged at 2800 R.P.M. for half an hour. The length of the column of bacteria deposited was measured to the nearest 0.1 mm. with a square jawed micrometer caliper, placing a plane mirror behind tube and caliper to obtain greater accuracy in reading. It was found that the ratio between the lengths of bacterial deposits determined after centrifuging for half an hour remained practically unchanged, even when centrifuging was continued for 45 minutes and 1 hour. Chilled samples of the cultures were diluted with a solution of methylene blue (0.1 cc. saturated alcoholic solution of the dye in 10 cc. saline) and counted in a bacterial counting chamber. The bacteria in 100, 1/400 mm. squares were enumerated. From the data thus obtained the relative volumes of equal numbers of organisms of the cultures to be compared were calculated. Duplicate determinations made on the same day, using the same culture, usually agree closely. Occasionally, measurements made on different days may show appreciable variation, probably due to the inherent difficulty of obtaining identical conditions of growth in different experiments. In order to minimize such discrepancies, the average of several determinations at each time interval has been taken.

Acid Agglutination.—Dextrose serum broth cultures of varying ages were centrifuged and the deposits taken up in sufficient saline to give whenever possible approximately the same density of suspension. To 0.25 cc. of a series of buffers varying from pH 1.1 to pH 6.0 was added 0.1 cc. of suspension. Sørensen's glycoll-hydrochloric acid buffers were used for the range pH 1.1 to pH 2.3, and

conversion was effected in the presence of killed smooth organisms from cultures of the rabbit virulent or avirulent strain. Below are presented the methods and the results of the experiments designed to answer these questions.

Technique

Cultures.—The two cultures, SV and CH, studied throughout the course of this work have been previously described (1). Rabbits survive the intravenous injection of 10 cc. of a 14 hour dextrose serum broth culture of CH, while the injection of 0.1 to 0.01 cc. of SV will bring about the death of the animal. Since CH grows but not as well at 41°C. as at 37°C., the experiments have been carried out at 37°C. to eliminate the factor of unfavorable influence of the higher temperature. Although the experiments reported here have been limited to only two of the strains studied in our first paper, our observations suggest that the findings apply also to the other strains.

Media.—The fluid medium adopted as standard for use throughout the experiments consisted of 100 cc. of sterile infusion broth to which was added 1 cc. of a sterile 10 per cent solution of dextrose in saline, together with 0.5 cc. normal rabbit serum. This medium was inoculated with 1 per cent of its volume of a 16 hour rabbit blood hormone broth culture, seeded with material from the stock cultures maintained in the manner already noted. In the study of colonial morphology, the horse blood neopeptone glycerin agar recently described by Ward and Lyons (4) was used.

Preparation of Antisera.—No great difficulty was encountered in producing in rabbits antisera of adequate titre against *Pneumococcus* Type III. 18 hour cultures in 0.1 per cent dextrose infusion broth of the strains CH and SV were centrifuged and the bacterial deposits taken up in a volume of 0.3 per cent formalin in saline equivalent to one-half that of the discarded supernatant, allowed to stand for 72 hours at 37°C. and then tested for sterility. Immunization of rabbits was carried out according to the method of Cole and Moore (5), using as the daily dose 0.5 cc. of vaccine. It was usually found necessary to administer three to five weekly courses of injections before securing a sufficient quantity of type specific agglutinin and precipitin. The anti-R sera were obtained in the same manner, using a rough variant derived from *Pneumococcus* Type I, with the exception that the vaccine was prepared without the use of formalin by heating the organisms for 20 minutes at 56°C.

Agglutination and Absorption of Agglutinins.—Unless a different procedure is described in the text, the antigens for agglutination were prepared by centrifuging the organisms from 15 hour broth cultures and resuspending the deposit in the original volume of fluid with 0.3 per cent formalinized saline. In testing for agglutinins, equal portions of the bacterial suspension and serum dilutions were mixed, left for 2 hours at 37°C. and overnight in the ice box before reading. In the experiment demonstrating the absence of a distinctive agglutininogen in

strain SV, 0.5 cc. quantities of the antisera used were twice absorbed with the deposit from 100 cc. of 0.1 per cent dextrose infusion broth culture, allowing a period of exposure to each absorbing dose of 1 hour at 37°C. 0.5 cc. of the antisera employed in demonstrating the similarity of old cultures of CH and SV to R variants in their agglutinative behavior were absorbed twice with the deposits from 20 cc. of 5 and 10 hour dextrose serum broth cultures of CH and the R variant respectively.

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McIlvaine's disodium phosphate-citric acid mixtures for the range pH 2.2 to pH 6.0. The pH of each buffer was checked potentiometrically. After being left 30 minutes at 37°C. and overnight in the ice box, readings were taken.

Phagocytosis.—The procedure employed in experiments involving the use of polymorphonuclear leucocytes in normal adult human defibrinated blood was essentially that described by Ward and Enders (7). Broth cultures were centrifuged and the sediment resuspended in sufficient broth to concentrate the numbers of pneumococci severalfold. In phagocytic experiments involving the use of rabbit leucocytes and serum the technique given by Enders and Wu (8) was adopted.

Method of Producing the R Variants.—1 drop of 18 hour blood broth cultures of CH and SV respectively was inoculated into 5 cc. of beef infusion broth containing 10 per cent antipneumococcus Type III rabbit serum (agglutinating titre 1/190 against 20 hour cultures of homologous strain CH) and incubated at 37°C. Thereafter, daily transfers were carried out in antiserum broth, using 1 drop as the inoculum. By the fifth transfer some R colonies were observed on plating out the cultures of both strains on 5 per cent horse blood infusion agar. All the colonies developing from the eighth transfer showed R characteristics. In each case, one colony was fished to blood broth. From the resulting cultures five daily transfers in this medium, as well as corresponding platings, were carried out; in none of the latter were S colonies observed. From the fifth such transfer single colonies were again fished and these cultures (designated CH-R and SV-R) of the two strains preserved for use. 0.7 cc. of either R culture was not fatal to mice on intraperitoneal injection.

EXPERIMENTAL

Cultural Characteristics

Growth in Fluid Media.—From data obtained by plating serial dilutions of samples taken at intervals from both CH and SV cultures when grown in dextrose serum broth at 37°C., it was possible to construct growth curves. These (Text-fig. 1) showed that the duration of lag and rate of multiplication in the logarithmic growth phase were nearly the same for these two strains. The time at which the maximum was reached was slightly later and the maximum numbers some 8 to 10 times less in the case of SV than for CH, both in dextrose serum broth and in blood broth. In defibrinated rabbit blood at 37°C. similar curves were obtained, with the maximum numbers for each being approximately equal. We wish to emphasize these similarities as being of basic importance since they indicate that the differences described in succeeding sections are not merely apparent and due to any prolonged lag of SV but represent real attributes which form distinguishing characteristics between the two strains even when they are in the same phase of growth.

The two R forms also exhibit distinctive properties when grown in blood broth. Here CH-R produces a diffuse clouding of the medium in contrast to the formation

of moderate sized granules by SV-R. If rapid transfers of the latter are carried out, it can be induced to grow diffusely; nevertheless, when the diffusely growing culture is plated on horse blood agar and again cultivated in broth, the granular type of growth is resumed. The SV-R growth in broth is likewise less luxuriant than that of CH-R.

Growth on Solid Media.—The two smooth strains cannot be differentiated on the usual 5 per cent horse blood infusion agar, since both form the large mucoid lenticular colonies typical of *Pneumococcus* Type III. On the neopeptone glycerin horse blood agar of Ward and Lyons after 16 hours, the CH colonies appear definitely whiter in comparison with the delicate pearl grey of SV. After 44 or more hours of incubation the flat CH colonies show dull granular surfaces with multiple small shiny prominences resembling tiny daughter colonies, contrasting with the dull smooth surface of SV colonies which is only rarely broken by a small papular eminence. The rough variants differ when grown on the latter medium. At 20 to 24 hours, CH-R is distinguished by a central portion much rougher in appearance than that of SV-R. After 48 hours the CH-R colonies (1.25 mm. diameter) are low convex or flat in shape. The surfaces, which fail to reflect the light, are covered with multiple papillae; the edges are smooth. At this time, optimal for differentiation, the slightly smaller SV-R colonies (1 mm. diameter) are slightly conical in shape, with a finely granular surface and irregular edges.

Evidence for the Identity of Antigenic Structure

Before proceeding further we considered it necessary to obtain evidence of the antigenic identity of the two strains. To this end, portions of antisera prepared against CH and SV were absorbed with the homologous and heterologous strain. To dilutions of these, as well as the unabsorbed sera, formalinized saline suspensions of the two strains were added. The results showed that there occurs mutual cross-absorption of the agglutinins produced in response to the injection of either organism into rabbits. Although this experiment does not completely eliminate the possibility of the existence of a distinguishing antigen situated deeply within the organism, it indicates at least that the more superficial antigenic layers primarily concerned in rendering the organism invulnerable to the defenses of the host are identical for the two strains.

Variation in Size of Capsule during Growth

The size and character of the CH and SV capsules were studied in stained smears during the first 34 hours of growth in broth at 37°C. From an examination of the series of photographs of such preparations reproduced in Figs. 1 to 20 it will be seen that after 3 to 4 hours incubation both strains possess well developed

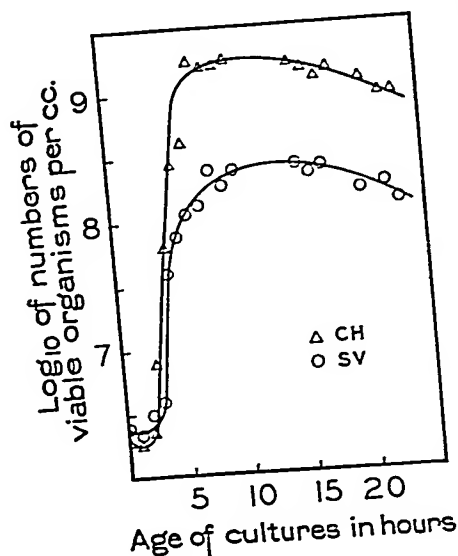
capsules, whereas the organisms in the original inoculum were noncapsulated. The envelope surrounding CH cells is, however, in most cases, somewhat smaller and does not stain so well or appear so dense as that of SV. On further incubation, the CH capsule becomes rapidly reduced and after 7 to 8 hours nearly all of the organisms have no stainable capsular material, although a few may still retain traces. At 10 hours no vestige is demonstrable. On the other hand, the majority of the individuals of SV retain even after 14 hours very large capsules with a periphery which takes the stain well. By 16 hours increasing numbers of decapsulated forms are seen, but it is not until 24 hours that practically every coccus appears devoid of capsule. We have noted that the dimensions of the cocci exclusive of the capsule are slightly greater in young than in old cultures, but have not studied this aspect of changes in size more closely.

We have also followed by means of stained smears the changes occurring when the two strains are grown at 37°C. in fresh normal rabbit serum or defibrinated rabbit blood, by inoculating 3 or 4 cc. of either of these media with 1 per cent of its volume of a 16 hour blood broth culture. In serum the CH cocci tend to grow in short chains, around which narrow faintly staining capsules are first definite after 3 hours, while the SV organisms appear chiefly as diplococci with large intensely stained capsules even after 1 to 2 hours. After 6 hours incubation only faint traces of fuzzy and uneven capsular material are observed in CH cells, while the SV organisms still show relatively large intact capsules after 13 hours. In blood the results are similar.

Since observed differences by the smear method might be exaggerated by artefact and, since through its use quantitative information concerning the relative size of the organisms could not be readily obtained, the results were confirmed and extended by measurement of the volume occupied by the bacteria at different times during growth. The relative sizes have been determined: (a) by comparing with 4½ hour CH as standard, the volumes occupied by equal numbers of the two strains grown in dextrose serum broth for periods varying usually by 2 hour intervals; (b) by ascertaining the ratio of the volume occupied by SV organisms to that occupied by equivalent numbers of CH of the same age during a period of incubation extending from 6 to 18 hours.

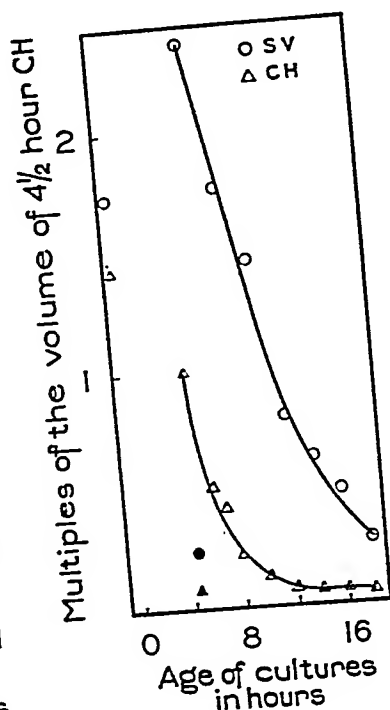
It has been impractical to secure determinations of the volume of CH before 4½ hours or SV before 6 hours, owing to the paucity of growth, while after 18 hours a tendency to clumping makes it difficult to enumerate the organisms in the bacterial counting chamber. From Text-fig. 2 it will be seen that the average volume of the SV cocci at 6 hours is 2.5 times that of CH at 4½ hours. Were it possible to secure sufficient numbers of each strain at a still earlier period this difference might possibly be even less, for on the basis of the smears we know that by 4½ hours many of the CH organisms have lost some of their capsules. There is no reason to believe, however, that strain CH ever quite attains the size of SV, since, as shown by the points to the left of the curves in Text-fig. 2, even the volume of CH organisms removed from the peritoneal cavity of the mouse killed 20 hours after infection is somewhat less than the volume of SV similarly obtained. Further

examination of this figure shows a rapid diminution in the volume of both organisms during the 6 to 12 hours incubation interval, with the average volume of CH after 10 to 12 hours having reached that of its R variant at $4\frac{1}{2}$ hours. In contrast, SV originally characterized by a larger capsule, requires more than 18 hours of growth to approximate the volume of its R derivative which at $4\frac{1}{2}$ hours is three to four times as large as CH-R.



TEXT-FIG. 1

TEXT-FIG. 1. Growth curves of *Pneumococcus* Type III, strains CH and SV, at 37°C. in dextrose serum broth.

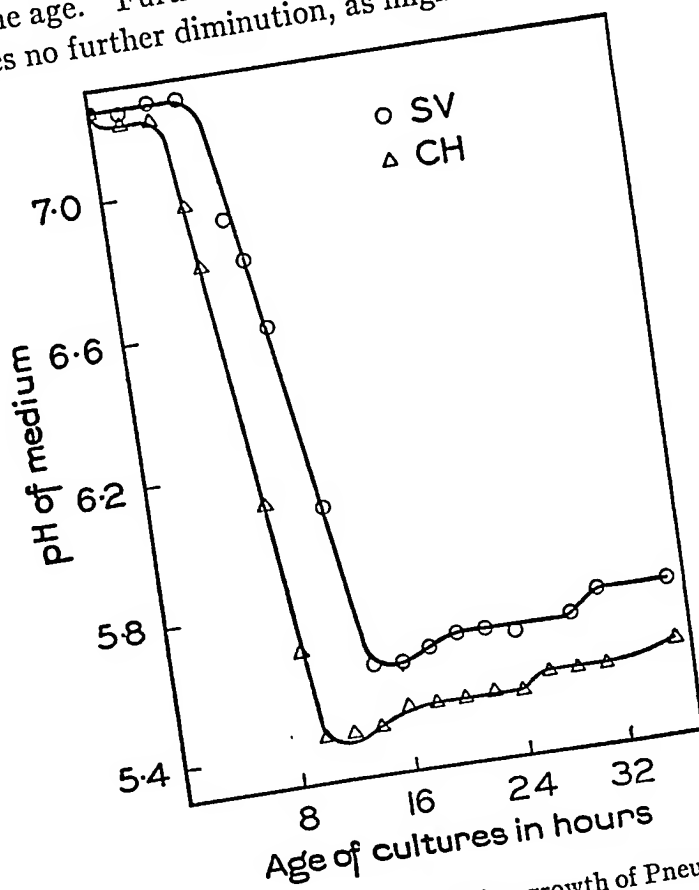


TEXT-FIG. 2

TEXT-FIG. 2. A comparison of the relative volumes of *Pneumococcus* Type III, strains CH and SV, in broth cultures of varying age with the volume of the organisms in a $4\frac{1}{2}$ hour culture of strain CH taken as unity. At the lower left are also indicated by solid black the volumes of the respective R variants at $4\frac{1}{2}$ hours. Points to the upper left of the curves indicate relative volumes of pneumococci taken from the peritoneum of the mouse.

In interpreting these results we consider that the alterations in volume represent chiefly changes in the quantity of capsular material

retained because of their agreement with the evidence offered by stained preparations and the fact that both encapsulated organisms ultimately are reduced to approximately the volume of their respective R forms which at 6 hours is about one-eighth that of the smooth cultures of the same age. Furthermore, the volume of CH after 10 to 12 hours undergoes no further diminution, as might be expected to occur



TEXT-FIG. 3. Changes in pH of medium during growth of Pneumococcus Type III, strains CH and SV, in broth cultures.

if the soma were shrinking, but remains practically unchanged in size. The disparity in size between the two R forms, which may also be demonstrated in stained preparations, is apparently not associated with any structure analogous to a capsule but is dependent upon the possession by SV-R of a greater quantity of somatic material. We thought it possible that the early loss of CH capsule might be effected by a greater and more rapid increase in the acidity of the

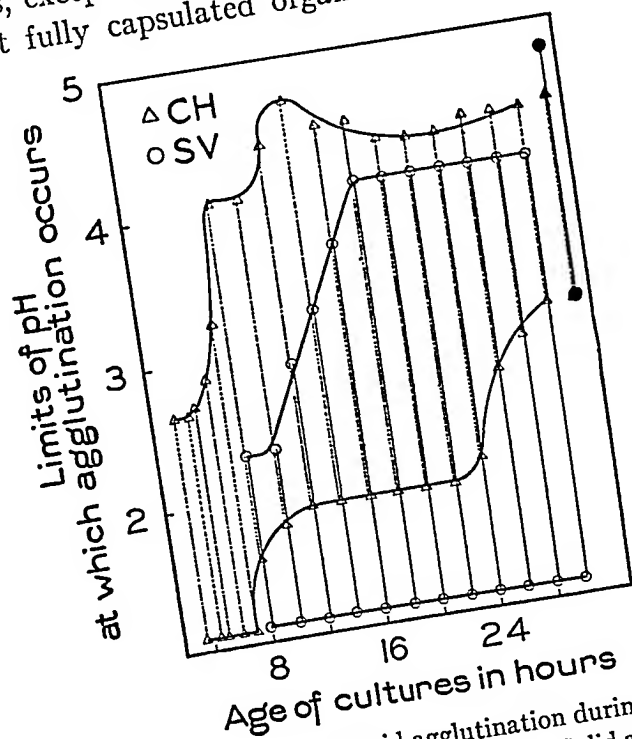
medium. Accordingly, potentiometric measurements of the hydrogen ion concentration were made on the culture medium at frequent intervals and curves constructed from the data. From Text-fig. 3 it is seen that in the CH culture the pH does fall sooner and reaches a slightly lower level. That the more rapid loss of capsule does not, however, depend on this increased acidity is demonstrated not only by the fact that the lowest pH is attained by SV at a time when it still possesses large capsules (12 hours), but also by experiments in which the reaction of the cultures was maintained at pH 7.2 during the same growth period through hourly additions of $N/1$ NaOH. No difference in capsular dimension was noted between smear preparations from the alkalized cultures and untreated controls. Furthermore, experiments have been performed in which cultures were acidified with $N/1$ HCl to pH 5.4 at a time when the organism possesses abundant capsule (after 4 hours incubation for CH; either 4 or 7 hours for SV). Smears were then made from time to time during continued incubation. It was found that even after 24 hours the acidified cultures had not lost their intact well developed capsules while the untreated controls showed none. Thus the presence of acidity equivalent to that developed during growth not only did not dissolve the capsular material but appeared to favor its retention. Although we have not been able to eliminate other unknown factors in the environment which might account for the difference in the rate of capsular loss, we believe on the basis of the available evidence that this is an inherent function of the individual strain.

Acid Agglutination at Various Periods

The striking diminution in volume led to the thought that definite alteration in the surface of the organisms should take place attendant upon the loss of capsular material. Thus substances of different chemical composition might be exposed which could shift the range of acid agglutination characteristic of the completely capsulated organism toward that in which clumping of the noncapsulated R variants occurs. Such displacement should be observed sooner in the case of CH.

In subjecting this hypothesis to experiment, cultures of CH and SV of varying age were centrifuged, resuspended in saline and added to

a series of buffers. Young cultures of the R variants of the two strains were likewise tested. The results are assembled in Text-fig. 4 in the form of a graph. It will be seen that in cultures of CH not more than $3\frac{1}{2}$ to 5 hours in age is the range of acid agglutination from pH 1.0 to pH 2.65, which corresponds to the range exhibited by SV even at 10 hours, except that the CH zone is slightly broader. We have found that fully capsulated organisms of both strains taken



TEXT-FIG. 4. Changes in the zone of acid agglutination during growth of broth cultures of *Pneumococcus* Type III, strains CH and SV. Solid and dotted vertical lines represent ranges of acid agglutination of strains SV and CH respectively. To the right of the curves are represented in solid black the zones for the R variants.

from the mouse peritoneum also agglutinate in the range pH 1.1 to pH 2.8. Beginning after 6 hours a swift displacement of the agglutinative range of CH occurs, which by 12 hours extends from pH 1.9 to pH 4.4 and which remains unchanged during the 10 hours following, except for slight fluctuations at the alkaline end. At 24 hours a second change begins at the acid end, causing a gradual contraction of the agglutination zone which at 30 hours becomes identical with that found for the homologous R form at $4\frac{1}{2}$ or 7 hours.

The behavior of strain SV is quite distinct. No change occurs until 12 hours when an extension of the alkaline end begins which takes place at a slower rate than the earlier rise of CH and attains a maximum only after 18 hours. Agglutination is found on the extreme acid end throughout the entire period of observation but although always marked, it may be incomplete in tubes of pH 1.1 to 2.0 after 16 to 18 hours or more of growth. The pH of the most alkaline buffer in which SV agglutinates is somewhat lower than that determined for the $4\frac{1}{2}$ or 7 hour homologous R form.

The use of buffers of pH as low as 1.1, which have not usually been employed in previous studies of acid agglutination, has revealed important differences in the behavior of the two strains. Gillespie (9) in his work on the acid agglutination of *Pneumococcus* Type I and III used lactate and acetate buffers ranging from pH 2.4 to pH 5.4 with cultures of 18 and 42 hours. He noted differences between these cultures in the hydrogen ion concentration producing agglutination. Hughes (10) also studied the agglutination of 8 hour cultures of typical and variant pneumococci in the buffer mixtures of Northrop and De Kruif, but observed no clumping of *Pneumococcus* Type III below pH 2.7. Presumably his culture was similar to CH. Sherman and Albus (11) found no agglutination of 4 hour cultures of *B. coli* in buffers of pH 3.0 to 4.1 to which the corresponding 24 hour cultures were susceptible.

Although Gillespie found that repeated washing does not appreciably alter the results, we have observed that with $4\frac{1}{2}$ to 6 hour CH cultures or suspensions from the mouse peritoneum, organisms washed one or more times with saline no longer agglutinate from pH 1.1 to 1.9, while SV organisms even in cultures as old as 16 hours remain unaffected by this procedure.

In all likelihood agglutination occurring from pH 1.1 to pH 2.3 or 2.6 is due to the material at the exposed surface of the capsule. Since the specific polysaccharide is an important constituent of the capsule it is not illogical to think that it may be the substance upon which this agglutination mainly depends. We have tested a sample of *Pneumococcus* Type III specific soluble substance, purified according to the procedure of Heidelberger, Goebel and Avery (12), by adding 0.1 cc. of an 0.08 per cent solution to a series of buffer tubes in the same manner as for acid agglutination. It was found that this SSS was thrown out of solution in the buffers of pH 1.1 to pH 1.5. A preparation of the

TABLE I

Agglutination of Strains SV, CH and a Rough Variant of Pneumococcus Type III in Type Specific and Anti-R Sera before and after Absorption

Agglutination of Strains SV, CH and a Rough Variant of Pneumococcus Specific and Anti-R Sera before and after Absorption									
Strain	Antiserum	Dilution of antisera							Remarks
		1/3	1/6	1/12	1/24	1/48	1/96	1/192	
CH 6 hrs.	Anti-S	—	++++±	+++++	+	0	0	0	Large flakes
	Anti-S absorbed with CH	—	0	0	0	0	0	0	Large flakes
	Anti-S absorbed with R III	—	+++	+++++	++	±	0	0	Medium size flakes
	Anti-R I	0	0	0	0	0	0	0	Medium sized flakes
CH 20 hrs.	Anti-S	—	+++++	+++++	+++++	+++++	+++++	++	Medium sized flakes
	Anti-S absorbed with CH	—	0	0	0	0	0	++	Small granules
	Anti-S absorbed with R III	—	+++++	+++++	+++++	+++++	0	—	Small granules
	Anti-R I	+++	+++	+	±	0	++	+	Small granules
CH 30 hrs.	Anti-S	—	+++++	+++++	+++++	+++++	+++++	++	Small granules
	Anti-S absorbed with CH	—	0	0	0	0	0	0	Small granules
	Anti-S absorbed with R III	—	+++++	+++++	+++++	+++++	±	±	Small granules
	Anti-R I	+++++	+++	++	—	—	0	0	Large flakes
SV 8 hrs.	Anti-S	—	+++++	++++±	±	0	0	0	Large flakes
	Anti-S absorbed with CH	—	0	0	0	0	0	0	Large flakes
	Anti-S absorbed with R III	—	+++++	+++	±	0	0	0	Large flakes
	Anti-R I	+	±?	0	0	0	0	0	Large flakes
SV 20 hrs.	Anti-S	—	+++++	++++±	+	0	0	0	Large flakes
	Anti-S absorbed with CH	—	0	0	0	0	0	0	Large flakes
	Anti-S absorbed with R III	—	+++++	+++±	±±	±?	0	0	Small granules
	Anti-R I	±	±	0	0	—	++	0	Small granules
SV 30 hrs.	Anti-S	—	+++++	+++++	+++++	+++++	+++++	0	Small granules
	Anti-S absorbed with CH	—	0	0	0	0	0	0	Small granules
	Anti-S absorbed with R III	—	+++++	+++++	+++++	+++++	+	0	Small granules
	Anti-R I	—	±	0	0	±	0	0	Small granules
R III 24 hrs.	Anti-S	—	+++++	+++++	+++++	+++++	0	0	Small granules
	Anti-S absorbed with CH	—	0	0	0	0	0	0	Small granules
	Anti-S absorbed with R III	—	+++++	+++	+++	++	0	0	Small granules
	Anti-R I	+++++	+++	++	++	++	0	0	Small granules

Incubate at 55°C. and overnight in the ice box.

Readings after one hour at 55°C. and overnight in the ice box.

± = agglutination.

0 = no agglutination.

— = not done.

± = trace of agglutination.

R III = the R form derived from strain SV. Anti-S = antipneumococcus Type III rabbit serum. Anti-S absorbed with CH = anti-S serum absorbed with organisms from a 5 hour dextrose serum broth culture of strain CH. Anti-S absorbed with R III = anti-S serum absorbed with a 10 hour dextrose serum broth culture of the R variant derived from strain SV. Anti-R I = antipneumococcus rabbit serum prepared by injection of an R variant obtained from Pneumococcus Type I.

specific carbohydrate purified in a different manner (Hornus and Enders (13)) precipitated only slightly in this pH range and then only after 2 to 3 days. We believe, however, it is the type specific substance upon which agglutination under these conditions depends and that the loss of the greatest proportion of its capsular substance by CH may account for the failure to agglutinate in buffers of pH 1.1 to 1.7 after 10 hours cultivation, whereas enough individuals of strain SV retain sufficient quantity of this material for as long as 20 to 30 hours to remain agglutinable in these concentrations of hydrogen ions.

If the curves for the acid agglutination values are compared with those shown in Text-fig. 2 it will be evident that the rapid loss in CH volume takes place synchronously with an equally rapid shift in the agglutinating range toward a more alkaline pH. Extension of the zone of SV agglutination in the direction of increased alkalinity begins only when the volume is reduced to an amount equivalent to that of CH at the time the latter exhibits an analogous agglutinative shift.

Agglutination in Type Specific Antiserum and Anti-R Serum

It seemed reasonable to think that the marked changes in susceptibility to clumping in buffers as growth proceeds denoted altered physicochemical properties of the bacterial surface which should be manifest when antigen was exposed to antibody. In Table I are recorded the results of an experiment in which the agglutinative behavior of living saline suspensions of both strains at different stages of cultivation was observed in the presence of anti-type specific and anti-R sera. They show that 6 hour cultures of CH which agglutinate in the anti-Type III pneumococcus serum fail to do so in anti-R serum, while 20 or 30 hour cultures are agglutinated in the latter serum to approximately the same titre as the R form. Nevertheless these older cultures are also clumped by type specific agglutinin, since absorption of the type specific antiserum with R organisms fails to remove this agglutinin which is completely taken out by a 5 hour culture of CH. Type specific agglutination in old cultures appears to be due to the very small quantities of the specific soluble substance too minute to greatly affect the zone of acid agglutination, but capable of uniting with homologous antibody. Marked reduction in the amount of type specific capsular material per organism also may account for the fact

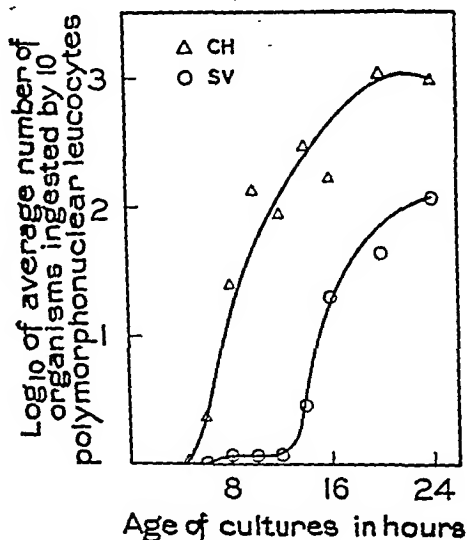
that the agglutinin titre of the anti-S serum against CH is about eight times greater if the antigen consists of a 20 or 30 hour culture than if a 6 hour culture is employed. We have confirmed this quantitative difference in the behavior of young and old cultures in similar experiments in which suspensions containing equal numbers of organism were used.

In general, SV agglutination in the various sera follows that of CH, but the changes occur at a later period. Thus in anti-R serum no definite agglutination is seen in 20 hour cultures. At 30 hours, however, in the anti-R serum it is agglutinated nearly to the titre of its homologous R derivative. No displacement of the end-point of agglutination in anti-type specific serum occurs with the 20 hour cultures while even with a 30 hour culture the titre of agglutinin appears only to be doubled in contrast to the apparent eightfold increase with the 20 hour CH culture. This disparity in the quantity of type specific agglutinin necessary to bring about equivalent effect in older cultures of SV may well be related to the hundred- to thousandfold difference noted by Watson and Cooper (14) in the quantity of protective substance required to save mice infected with the same doses of a rabbit virulent and a rabbit avirulent strain of *Pneumococcus* Type III. Finally we wish to point out that although a 6 hour CH culture fails to agglutinate in anti-R serum, it is nevertheless capable of binding agglutinin reacting with the R variant. Pertinent in respect to these findings are those of Tillett (15) who showed that chemically decapsulated *Pneumococcus* Type III agglutinated to the same titre as the R variant in anti-R serum.

Phagocytosis by Human Polymorphonuclear Leucocytes

The readiness with which strains CH and SV were taken up by the polymorphonuclear leucocytes in normal adult human defibrinated blood was determined for dextrose serum broth cultures of varying age. From Text-fig. 5 it will be observed that $4\frac{1}{2}$ hour cultures of strain CH are completely resistant to phagocytosis. As early as 6 hours a few organisms have reached a state such that they can be ingested. Thereafter a rapid rise in the number of intracellular cocci takes place. Sharply at variance with this swift loss of resistance to phagocytosis is the prolonged retention of the property by SV. Very few organisms less than 14 hours in age appear in leucocytes,

but at this time a slight increase in phagocytosis is observed which becomes greater with further cultivation. In the following paper observations concerning the phagocytosis of these strains by the leucocytes of the rabbit and mouse have been recorded. We may note here, however, that in a system of normal rabbit leucocytes and serum, active phagocytosis of both R variants occurred. In one experiment the number of cocci from a 6 hour CH-R culture ingested by ten polymorphonuclears was 385, while 108 SV-R organisms were



TEXT-FIG. 5. Phagocytosis by polymorphonuclear leucocytes in normal adult human defibrinated blood of *Pneumococcus* Type III, strains CH and SV, at intervals during growth in broth.

taken up by the same number of cells. Since in each case the leucocytes appeared to be completely filled with organisms, this difference may not represent any greater susceptibility to phagocytosis of CH-R, but probably depends on the greater somatic volume per coccus of SV-R.

Reconversion of the R Variants to S Forms

Employing the method of Griffith, the two R strains possessing the characteristics described above were converted to the S form under the following conditions: (a) injection into mice of a small quantity of

the living R form derived from either the rabbit virulent or avirulent strain, together with a large quantity of heat-killed S form of the homologous or heterologous strain; (b) injection of a large quantity of the living R form alone.

The killed S vaccines were prepared by centrifuging the deposits from 6 hour dextrose serum broth cultures of smooth strains CH and SV and resuspending in sufficient physiological saline to yield suspensions concentrated about 150-fold, which were heated at 60°C. for $\frac{1}{2}$ hour. Their sterility was controlled by inoculation of plates and broth as well as mice.

White mice weighing 15 to 18 gm. were injected subcutaneously with 0.5 cc. of these vaccines together with 0.5 cc. of a 15 hour blood broth culture of the R variant. Additional mice were injected subcutaneously with 0.5 cc. of broth suspension of the R variants representing the centrifuged deposit from 100 cc. of 15 hour blood broth culture. The combined details and results of two experiments are recorded in Table II.

From the cultures of the heart's blood of the mice dying as a result of the infection, typical smooth colonies were fished to blood broth and studied in respect to colony morphology on the Ward-Lyons medium, growth characteristics in broth, survival in defibrinated rabbit blood under vaseline seal at ice box temperature, capacity to resist the phagocytic action of normal human leucocytes and serum, and finally the virulence of certain selected ones for the rabbit.

Appearance of Colony Produced by Reconverted S Forms and Characteristics of Growth in Broth.—Broth cultures developing from the single colonies fished from the platings of the heart's blood of the mice were seeded onto the glycerin blood agar medium along with organisms from cultures of the stock strains of CH and SV. A careful study of the single colonies showed that in every case in which living SV-R had been injected either alone or together with heat-killed S, the colony morphology corresponded to that of the stock smooth SV, regardless of whether or not the vaccine (if employed) was prepared from this strain or from CH. On the other hand, in the animals which had received CH-R the smooth forms which developed appeared to be identical in respect to the colony with the stock CH even when produced under the influence of SV vaccine. From these observations, it is clear that when reversion to the smooth form took place the morphology of the colony was the same as that of the strain from which the R variant derived, and was not influenced by the origin of the vaccine employed as an agent in the process of conversion.

Colonies picked from the plate cultures used for the morphological study were inoculated into blood broth and the density of the growth noted. In this characteristic, also, the reconverted strains resembled the stock smooth strains, since cultures of organisms from reconverted CH-R, independent of the means used in effecting the transformation, with one exception grew luxuriantly, in contrast to those representing SV-R brought back to the S phase, all of which exhibited a more

delicate growth. In these studies 36 colonies were tested; two to six colonies were selected from the heart's blood culture of each mouse.

Survival of Reconverted R Variants in the Cold.—We had made the observation that under the conditions in which the stock strains were maintained (1), strain SV would survive for periods exceeding 18

TABLE II
Reconversion of R Variants of Pneumococcus Type III to the Smooth Form

Group	Materials injected	No. mice in each group	No. mice dying in each group and time of death	Results of cultivation of heart's blood	Results of cultivation of material from site of injection	Remarks
1	0.5 cc. CH vaccine	3	0	—	—	3 surviving mice killed after 2 wks. Cultures of heart's blood and injection sites were sterile
2	0.5 cc. SV vaccine	3	0	—	—	
3	0.5 cc. CH-R culture	3	0	—	—	
4	0.5 cc. SV-R culture	1	0	—	—	
5	0.5 cc. CH-R culture + 0.5 cc. CH vaccine	5	2—72 hrs.	Many R and a few S colonies	Many R and a few S colonies	
6	0.5 cc. CH-R culture + 0.5 cc. SV vaccine	5	3 { 1—48 hrs. 2—72 hrs.	All S, no R colonies	Many S and a few R colonies except in one case which gave all S forms	Cultures from survivors were sterile
7	0.5 cc. SV-R culture + 0.5 cc. CH vaccine	1	1—60 hrs.	All S, no R colonies	Many S and a few R colonies	Cultures from survivor killed after 2 wks. were sterile
8	0.5 cc. SV-R culture + 0.5 cc. SV vaccine	1	1—60 hrs.	All S, no R colonies	Many S and a few R colonies	
9	100 cc. CH-R culture	2	1—72 hrs.	All S, no R colonies	Not done	
10	100 cc. SV-R culture	2	2 { 1—72 hrs. 1—96 hrs.	1—S and R colonies 1—only S colonies	Not done	

months, while CH under the same conditions died out within 9 months to a year. With this in mind, the viability of a selected number of the cultures was tested after the cultures in defibrinated rabbit's blood under a vaseline seal had remained in the ice box for 16 months. Eight cultures representing the various combinations involving the

transformation of SV-R and seven cultures originally obtained from mice injected with CH-R were plated. All of the former were found to be viable, whereas none of the reconverted CH-R forms had survived, nor a culture of CH stock kept under the same conditions. In this respect then the S forms obtained from CH-R and SV-R behaved like the original smooth cultures, although in certain instances their reversion was mediated by vaccine derived from the heterologous strain.

Resistance to Phagocytosis of the Reconverted Strains.—Since it was found that there was a marked difference in the resistance of young cultures of the two stock smooth strains to the phagocytic action of normal human leucocytes, the effect of this system on 6 hour blood broth cultures of 32 of the 36 reconverted colonies noted above was studied. Upon testing these within 1 to 2 weeks following their transformation it was observed that in the case of cultures, emanating from both CH-R and SV-R that their resistance to phagocytosis was not identical with that of the smooth stock strains (Table III).

It is difficult to offer an explanation for this behavior. That it is however, at least in the case of the reconverted SV-R cultures, a transitory phase, representing possibly unstable properties on the part of some of the organisms which after a time become fixed according to the pattern of the stock strain, is demonstrated by the fact that after maintenance in defibrinated rabbit blood for 16 months in the ice box these, like the stock strain of SV, almost completely resist the phagocytic attack by the cells of the same normal human blood in which they were first tested. Since the cultures of reconverted CH-R colonies had died out during this time, it was impossible to determine their resistance to phagocytosis.

Virulence for Rabbits of the Transformed R Variants.—16 to 18 hour blood broth cultures obtained from certain colonies selected from those which were studied and which represent the various combinations utilized in transformation were injected intravenously into rabbits with the object of determining their virulence for those animals. The cultures of reconverted CH-R variants which were tested were those which were found to exhibit the greatest resistance to phagocytosis in the previous experiments. The experimental details are incorporated in Table IV. From those recorded for the first four animals it is

apparent that in every instance in which the rough variant was obtained from the rabbit virulent strain and again converted to the smooth form, the latter killed the rabbit, whatever the means employed for accomplishing the transformation. It thus appeared that the capacity of the transformed R variant of the rabbit virulent strain to produce in small amounts a fatal infection in rabbits was not

TABLE III

Phagocytosis in Human Blood of Organisms from 6 Hour Blood Broth Cultures of Reconverted and Stock S Strains

Source of culture	No. of colonies* studied	Average of phagocytic counts on cultures from colonies (No. cocci per 10 leucocytes)	Highest count (No. cocci per 10 leucocytes)	Lowest count (No. cocci per 10 leucocytes)	Remarks
Mice injected with CH vaccine and CH-R culture	6	45.4	88.5	15.5	3 colonies studied from each of 2 mice injected with this combination
Mice injected with SV vaccine and CH-R culture	9	51.2	82.5	20.5	3 colonies studied from each of 3 mice injected with this combination
Mouse injected with CH-R culture only	2	93.7	121.0	66.5	2 colonies studied from 1 mouse injected
Stock CH culture	—	74.9	—	—	
Mouse injected with SV vaccine and SV-R culture	6	24.6	36.4	18.6	6 colonies studied from 1 mouse injected
Same after 16 mos.	2	1.3	2.6	0.0	
Mouse injected with CH vaccine and SV-R culture	6	22.7	25.0	16.6	6 colonies studied from 1 mouse injected
Same after 16 mos.	2	0.0	0.0	0.0	
Mice injected with SV-R culture only	3	17.8	26.0	2.0	2 colonies studied from 1st mouse injected. 1 colony studied from 2nd mouse injected
Same after 16 mos.	2	1.05	1.1	1.0	
Stock SV culture	—	0.0	—	—	

*Only S colonies from hearts' blood cultures were studied.

influenced by the mode of reversion. We especially wish to emphasize the fact that although the vaccine from the rabbit avirulent strain affords the necessary stimulus for reversion of R to S, it induces no change in the direction of a decreased virulence on the part of the reconverted form.

Of the rabbits injected with cultures representing the smooth forms of the R variant derived from the rabbit avirulent strain one animal

died after 4 days and from the heart's blood a pure culture of pneumococcus was recovered. We do not regard this result as demonstrating an increase in virulence on the part of the reconverted organisms since a further examination of the data will show that two animals, one of which was inoculated with the same culture and another with the organisms grown from the heart's blood of the animal which died, both recovered after exhibiting a bacteremia in all respects comparable

TABLE IV
Virulence for Rabbits of S Strains Reconverted from R

Rabbit No.	Origin of culture*	Amount of culture injected intra-venously	Result		Remarks
			cc.	hrs.	
1	SV-R culture and CH vaccine	0.1		D 48	Heart's blood: Positive. Culture was the 4th daily transplant from original single colony fishing
2	SV-R culture and CH vaccine	0.1		D 96	Heart's blood: Positive. Same culture as used in rabbit 1 but preserved in defibrinated blood for 16 mos.
3	SV-R culture and SV vaccine	0.1		D 27	Heart's blood: Positive. Culture was the 4th daily transplant from single colony fishing
4	SV-R culture only	0.1		D 60 ±	Heart's blood: Positive. Culture had been preserved 16 mos. in defibrinated blood before testing
5	CH-R culture and SV vaccine	0.2		S	Culture obtained from original colony No. 10 fishing; preserved for 2 wks. in defibrinated blood
6	CH-R culture and SV vaccine	0.2		D 96	Heart's blood: Positive. Culture obtained from original colony No. 17 fishing; preserved for 2 wks. in defibrinated blood
7	CH-R culture and SV vaccine	0.2		S	Culture from original colony No. 17 fishing. Blood cultures at intervals gave curve typical of strain CH
8	CH-R culture and SV vaccine	0.2		S	Culture from heart's blood of rabbit 6 used for injection. Blood cultures at intervals gave curve typical of strain
9	CH-R culture only	0.2		S	Culture from original colony fishing; preserved for 2 wks. in defibrinated blood

* 17 hour blood broth cultures of S colonies obtained from cultures of heart's blood of mice injected with the combinations noted.

to that following the injection of strain CH. It is probable that the death of this animal was due to some other cause at a time when the low grade bacteremia characteristically produced by the avirulent form was still present. If, then, this explanation be correct, it may be stated that the R variant obtained from the rabbit avirulent strain and transformed again to the S under the influence of a vaccine prepared from the virulent strain does not exhibit any increased virulence but behaves in this characteristic like the rabbit avirulent strain from

which it originated. In addition, it may be noted from Table IV that this same R variant when injected in large quantity into mice produces an S form which is avirulent for the rabbit. Cultures of the CH-R variant reconverted in the presence of the homologous smooth vaccine were not tested for their rabbit virulence since there was no reason to believe that any difference from the parent strain would be observed.

SUMMARY AND DISCUSSION

The results which have been presented show that under the conditions of artificial cultivation at 37°C. definite differences exist between two smooth strains of *Pneumococcus* Type III both of which are highly virulent for mice by the intraperitoneal route, but which may be sharply distinguished in their virulence for rabbits. These differences consist in the size of the fully developed intact capsule and the interval of time required for its loss. The somewhat smaller capsule of the avirulent strain, well formed and easily demonstrable during the early period of growth, diminishes quickly, while the large capsule of the strain virulent for rabbits is retained for a considerably longer period. Closely correlated with the time at which this reduction of capsule occurs is the appearance of changes in the surface properties of the bacteria which are revealed by a shifting of the range of acid agglutination, susceptibility to clumping in anti-R serum and ingestion by normal adult human polymorphonuclear leucocytes and serum. Since it has been shown that these alterations as growth continues, result in a loss of characteristics which distinguish the strictly type specific, fully capsulated pneumococcus and ultimately lead to a state temporarily approximating that of the completely avirulent R form, and since under the experimental conditions they are inaugurated sooner, advance more rapidly and are more complete in the rabbit avirulent organism, we believe that they may partly account for difference in rabbit virulence of the two strains. In the following paper an attempt has therefore been made to correlate this behavior *in vitro* with the events attendant upon inoculation into the animal body.

The studies of Clark and Ruehl (16), Henrici (17), Bayne-Jones and Adolph (18) and others have demonstrated a marked increase in the size of the bacterial cell associated with the early phases of growth.

These authors have dealt chiefly with noncapsulated rod forms and even Clark and Ruchl who included cultures of various cocci do not make reference to variations in capsule size. Recently Seastone (19) has called attention to the large volume occupied by young capsulated streptococci. Similarly we have found that increase and decrease of *Pneumococcus* Type III volume appears to be due largely to the formation of capsule in young cultures and its subsequent loss as the organisms age. Because of the relatively great proportion of capsule in comparison with soma, a greater disparity exists between the volume of young and old pneumococci than that found by those who have studied bacteria lacking this structure. Of interest in connection with our observations are those of Preisz (20) on the nature of capsules of virulent anthrax bacilli and strains attenuated by cultivation at 42.5°C. The latter produced soft, rapidly dissolving capsules while such structures in the former were characteristically firm and were retained by the bacilli for longer periods. This worker also noted in confirmation of the earlier work of others, that the capsules of *B. anthracis* are lost during the course of growth in serum media and in the subcutaneous tissues of the susceptible mouse.

We have demonstrated that the R variants derived under the same conditions from the two smooth strains of *Pneumococcus* Type III reveal certain characteristics by which they may be distinguished from each other in respect to cell and colony morphology, growth in broth, as well as growth at 41°C. (*cf.* Paper I). By employing the method of Griffith, these two R variants have been induced to revert to the S form. Following the injection into mice of the various possible combinations of living R variant and the killed S organisms of either rabbit virulent or avirulent strain, as well as very large numbers of the R variant alone, S forms emerged which in their various attributes, notably that of virulence for rabbits, resembled the original smooth strain from which the particular R variant involved was dissociated. The function of the smooth killed organisms in the process of transformation appeared to be only that of a stimulus toward reversion to the S. They apparently play no rôle in determining the virulence or the growth properties of the resulting S form. These observations indicate that the factors involved in virulence are conditioned by stable physiological properties peculiar to the

individual strain and that although temporarily inactive during the R state, they are again resumed unaltered upon the transition to the S form. They serve also to reemphasize the fact, apparent from several studies but perhaps not sufficiently realized, that the R variants of the pneumococcus, even though obtained under the same conditions from the same type but from different strains, may vary definitely in their various attributes.

Finally, they strongly suggest that the degree of virulence of a given strain of a bacterial species may be determined not only by its ability to multiply in the environment of the host and to synthesize certain substances of definite chemical and antigenic properties, but also by the capacity to elaborate these in greater or lesser degree and under the conditions of parasitism within the animal body to maintain them in contact with the soma of the cell in such state that they afford an efficient barrier to the defensive mechanisms of the host.

CONCLUSIONS

1. Certain properties exhibited in culture by two smooth strains of Pneumococcus Type III, one virulent for rabbits and the other relatively avirulent for these animals, have been studied. No antigenic differences between these strains have been revealed by the cross-absorption of agglutinins from homologous antisera.

2. In young cultures the organisms of both strains possess well developed capsules. It has been shown, however, by stained preparations and measurements of relative volume, that the rabbit avirulent strain loses this capsule in dextrose serum broth cultures about 8 hours before it disappears in the case of the rabbit virulent organism. Similar results have been obtained when growth takes place in normal rabbit blood or serum. The rate at which loss of capsule occurs is independent of increased acidity attendant on growth.

3. Loss of capsule in both cases goes *pari passu* with marked shrinkage in volume, alterations in the zone of acid agglutination, susceptibility to agglutination in anti-R pneumococcus serum and to phagocytosis.

4. Because of these changes, older cultures of both strains appear to be composed largely of individuals which approximate the avirulent R form in respect to size and surface properties. In the rabbit avirulent

strain this approach to a state analogous to that of the R form begins earlier, proceeds more rapidly and goes further toward completion.

5. R variants dissociating from these two smooth strains of Pneumococcus Type III are distinguishable from one another in morphological and growth properties.

6. When reversion to the smooth type specific form of such R variants occurs, the organisms closely resemble in respect to the virulence for rabbits and other attributes the original strain from which the R variant was derived, regardless of whether killed organisms of the rabbit virulent or avirulent strains were employed in effecting the reversion.

7. On the basis of these findings it appears that the factors upon which the difference in virulence between two strains of smooth Type III pneumococcus may depend are the products of stable physiological processes of the bacterial cell which are retained during the transformation $S \rightarrow R \rightarrow S$.

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EXPLANATION OF PLATE 20

The photographs were made from smears of dextrose serum broth cultures of *Pneumococcus* Type III, strains SV and CH, stained with the alcoholic methyl violet-basic fuchsin stain described in the text. Figures to the right of the black line are of strain CH, those to the left of strain SV.

FIGS. 1 and 2. Strain SV. 3 hour culture. Note completely developed capsule.

FIG. 3. Strain SV. 4 hour culture. Large intact capsule.

FIG. 4. Strain CH. 4 hour culture. Large intact capsule.

FIGS. 5 and 6. Strain SV. 6 hour culture. Capsules.

FIGS. 7, 8 and 10. Strain CH. 6 hour culture. Note diminished and imperfect capsule in the case of certain organisms and its absence in others.

FIG. 9. Strain SV. 10 hour culture. Large intact capsule. Compare with Fig. 15.

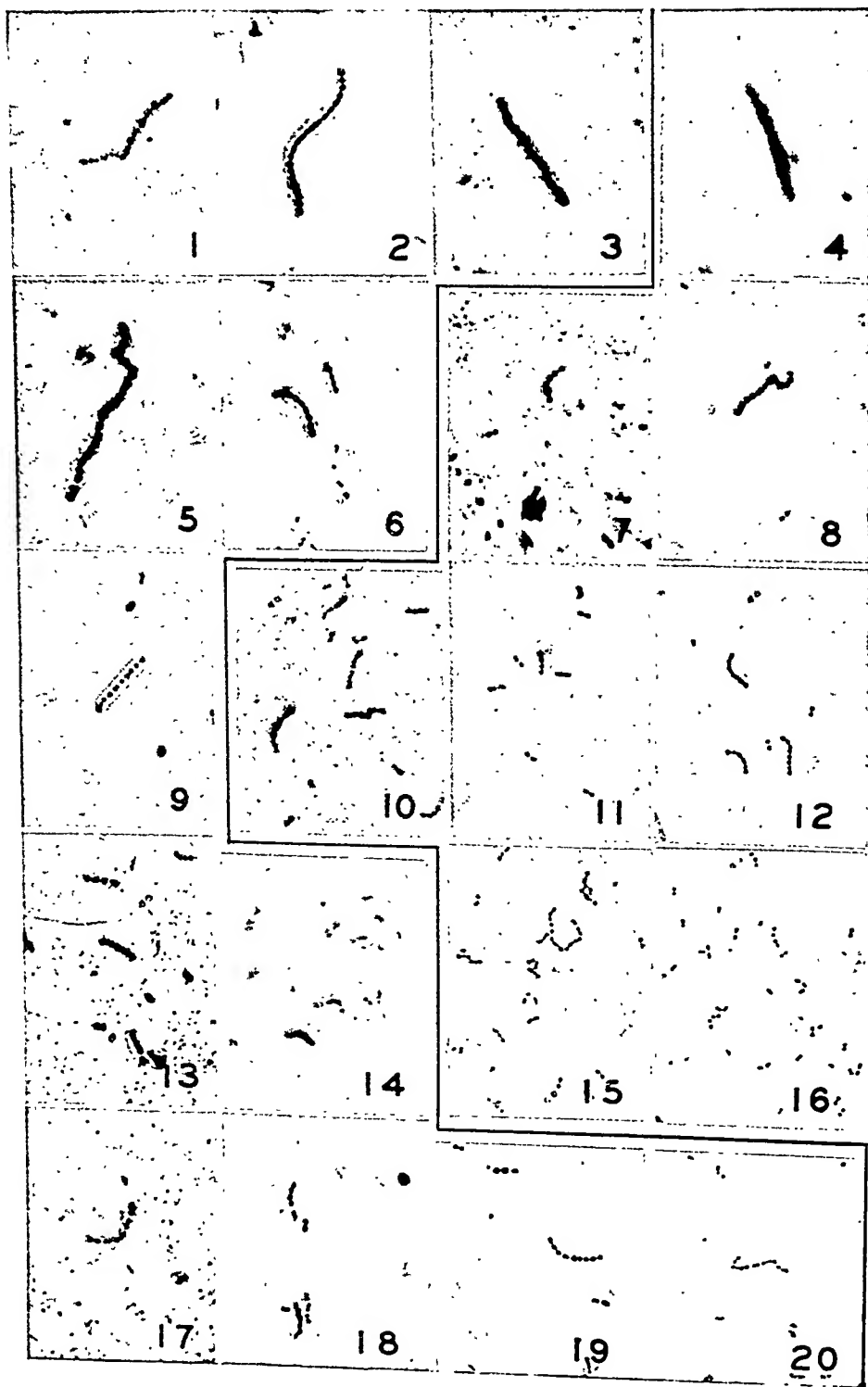
FIGS. 11 and 12. Strain CH. 7 hour culture. Note the almost complete absence of capsule.

FIGS. 13 and 14. Strain SV. 14 hour culture. Capsules are still present.

FIGS. 15 and 16. Strain CH. 10 hour and 14 hour cultures. Complete absence of capsules.

FIGS. 17 and 18. Strain SV. 16 hour culture showing in one instance large capsule, in others only traces of the structure.

FIGS. 19 and 20. Strain SV. 22 hour culture. Complete absence of capsules. Compare with Fig. 15.



STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

III. CORRELATION OF THE BEHAVIOR IN VIVO OF PNEUMOCOCCI TYPE III VARYING IN THEIR VIRULENCE FOR RABBITS WITH CERTAIN DIFFERENCES OBSERVED IN VITRO

BY JOHN F. ENDERS, PH.D., MORRIS F. SHAFFER,* PH.D., AND
CHAO-JEN WU,* M.D.

(From the Department of Bacteriology and Immunology, The Harvard Medical
School, Boston)

PLATE 21

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In an accompanying paper it has been shown that two strains of *Pneumococcus* Type III differing in rabbit virulence could be distinguished in respect to the rate at which the capsule diminished during cultivation in various media. Concomitantly with the progressive loss of capsular substance, changes in the surface properties of the organisms were demonstrated which ultimately, but after different periods, reduced each strain to a state in many respects resembling that of the R form. The principal objective of the experiments to be presented here was to reveal a relationship between these facts and the events which ensue when the organisms are introduced into the animal body.

Materials and Technique

Strains of Pneumococci.—The two strains of *Pneumococcus* Type III designated SV and CH, and the R variant derived from each as described in Paper II were used throughout these experiments, as well as a stock R derivative from *Pneumococcus* Type I.

Animals.—In all cases, unless specific descriptions are given in the text, albino rabbits ranging in weight from 1600 to 2000 gm. and white mice of 15 to 20 gm. weight were used.

* At the time this work was done Dr. Wu held a Travelling Fellowship from the Peiping Union Medical School, and Dr. Shaffer a Fellowship in the Medical Sciences from the National Research Council.

Media.—Cultures of all the strains of pneumococci were regularly grown in the rabbit serum dextrose infusion broth medium described in Paper II, except in certain cases noted in the text. Seed cultures and stock cultures were grown in the same media as previously described.

Blood Cultures.—Blood was removed from the ear vein of rabbits into a tuberculin syringe and measured portions of serial dilutions plated in Petri dishes containing 0.5 cc. of defibrinated horse blood and melted infusion agar.

Phagocytosis.—

1. *In Vitro.*—In examining the phagocytic properties of rabbit leucocytes mixed with rabbit defibrinated blood, the method of obtaining and standardizing the materials was that of Robertson and Sia (1) as modified by Enders and Wu (2) for use in bactericidal measurements, with the exception that leucocytic exudate was produced within the peritoneum by means of large injections of saline, instead of in the pleural cavity. The cells and serum of mice were obtained in the following manner: 24 hours before the experiment, normal mice were injected intraperitoneally with 1.0 cc. of physiological salt solution and again with the same quantity after 22 hours. 2 hours later, the animals were anesthetized with ether and bled from the heart, using a tuberculin syringe and a 27 gauge needle. The blood, usually amounting to about 1 cc., was defibrinated in a test tube with a few glass beads. The peritoneum was then washed out with a small quantity of 1.5 per cent citrate solution. After twice washing the cells thus obtained in saline, the defibrinated blood from the same animal was added to them. They were then gently mixed and used in the same way as that of the similar combination of rabbit cells and serum.

2. *In Vivo.*—An exudate of leucocytes was induced in the pleural cavity of rabbits by the injection of 4 cc. of sterile infusion broth. 20 hours later, 0.2 cc. of thick suspension of the organisms centrifuged from 10 cc. of 23 hour broth cultures was injected. At intervals thereafter, samples of exudate were aspirated into a tuberculin syringe. Smears of the exudate were stained with Wright and Gram stains. For the latter, the preparation was fixed for 1 to 2 minutes in methyl alcohol. 2 per cent aqueous neutral red was employed as a counterstain. Measured volumes of exudates were diluted, and plates poured according to the procedure described for blood cultures.

For observing the phagocytosis within the peritoneal cavities of mice, 0.5 cc. of sterile saline was administered twice intraperitoneally into a series of mice at 16 to 18 hour intervals. 4 hours later, 0.1 cc. of organisms resuspended in sterile broth after centrifugation of cultures of various ages was injected. At subsequent intervals, a mouse was sacrificed and smears made from the peritoneal exudate.

In studying the phagocytosis accomplished by the fixed tissue cells of the rabbit, the bacteria from 20 to 80 cc. of blood broth cultures of pneumococci were injected in 1 to 2 cc. of sterile broth. After the sacrifice of the animals at varying periods subsequent to infection, portions of various organs were fixed in Zenker's solution. From these sections were prepared and stained with Gram-Weiger and Giemsa stains.

Technique Employed in Estimating Number of Viable Organisms in Various Tissues.—At intervals after intravenous infection of rabbits, portions of the various organs were removed and their weight determined to the nearest milligram, then ground for about 10 minutes in a mortar containing sterile sand. To the tissue sufficient sterile broth was added to give a 1/50 suspension, from which serial dilutions were then prepared, and 0.1 cc. portions plated according to the method used for culturing the blood.

EXPERIMENTAL

The Correlation of Capsule with the Initial Clearing of the Organisms from the Blood Stream

Following the intravenous inoculation of 12 to 14 hour broth cultures into rabbits, Tillett (3) has demonstrated a difference in the behavior of rabbit virulent and avirulent strains of *Pneumococcus* Type III. The organisms of the former strain either remained in the blood stream in large numbers or underwent a certain degree of initial removal. After a stationary period, they began to increase steadily, followed by the death of the animal. On the other hand, with the avirulent strains the numbers in the blood rapidly underwent a marked reduction with a subsequent moderate increase after several hours. Thereafter, a low fluctuating degree of bacteremia was maintained for 4 or 5 days, followed by recovery. In the experiments which follow, we have employed the same method, except that more exact estimates of the numbers of organisms present in the blood were obtained in studying the rate at which removal of the rabbit virulent strain SV and the rabbit avirulent strain CH took place when cultures of various ages were injected. The results, when broth cultures of the age (12 to 14 hours) employed by Tillett were injected, agree in general with his. When, however, still older broth cultures, or young, encapsulated organisms or "animal" bacteria were introduced into the blood stream, a very different type of initial elimination curve was secured. In Text-figs. 1 and 2 are presented two series of curves obtained by plotting the logarithms of the number of organisms of strains CH and SV per cubic centimeter of blood at different intervals during the period immediately subsequent to infection. Each curve represents the course of the bacteremia in a rabbit inoculated with one of the following: a culture of varying age, mouse peritoneal washings or the blood of an infected rabbit taken shortly before death. The experimental data used in drawing the curves, as well as additional material, are presented in Tables I and II. It will be seen that the organisms derived from the animal body (mouse peritoneum or blood from an infected rabbit) or from very young (3 hour broth) cultures of strain CH remain in large numbers in the blood during the first 4 hours of experiment. The aging of the CH culture during artificial cultivation, however, very rapidly induces a change in the organism which is reflected in the increased rate of removal from the blood. Thus, the numbers of a 4½ hour broth culture are reduced about one hundredfold within 40 minutes. After 5 hours growth in broth, the organisms have become so altered that at the

IMMUNITY TO PNEUMOCOCCUS TYPE III. III

end of 30 minutes only about 1/10,000 of those present after 1 minute are found in the blood. 10 hour broth cultures of this strain disappear so swiftly that after 1 hour only 10 organisms or less per cc. of blood are found.

In general, the curves obtained with comparable cultures of strain SV follow the same spatial pattern. The majority of animal organisms

TABLE
Intravenous Inoculation of N. l.
Results of Blood Cultures (Expressed as Numbers of V.C.)

Time interval elapsing between infection and blood culture	Organisms from mouse peritoneum				Org. died
	Rabbit 8 (Text-fig. 1, curve A)	Rabbit 10	Rabbit 21	Rabbit 17	
min.					
1	3.6×10^7	3.9×10^7	2.9×10^8	1.5×10^8	
10	2.5×10^7	3.0×10^7	4.7×10^7	1.7×10^8	
20	3.4×10^7	1.4×10^7	3.2×10^7 (40 min.)	1.3×10^8	
30-40	1.1×10^7 (40 min.)	7.3×10^6 (40 min.)		1.6×10^8 (40 min.)	158
hrs.					
1	1.3×10^7	2.6×10^6	2.3×10^7	3.5×10^7	
2	6.7×10^6	8.3×10^3	5.8×10^5	1.2×10^8	11
3	—	—	1.6×10^4	5.6×10^7	4.7
4	1.9×10^5	2.0×10^3	2.9×10^5	—	
5	—	—	5.0×10^4	—	
16-20	2.5×10^7 (18 hrs.)	2.0×10^3 (23 hrs.)	3.0×10^5 (22 hrs.)	2.0×10^8 (19 hrs.)	
21-25	—	—	9.2×10^3 (26 hrs.)	2.9×10^8 (22 hrs.)	
26-40	—	—	1.4×10^3 (28 hrs.)	—	
41-52	—	200 (46 hrs.)	2.2×10^3 (47 hrs.)	—	
69-72	—	23 (72 hrs.)	100 (52 hrs.)	—	
92-97	—	0 (97 hrs.)	0 (72 hrs.)	—	
116-140	—	—	0 (96 hrs.)	—	
Final outcome.....	D in 24 hrs.	R	R	D in 23 hrs.	

D = died.

R = recovered.

and those from younger cultures remain in the blood and within a short time bring about a fatal outcome, whereas those grown for a longer period in broth previous to injection are quickly removed, although they ultimately reappear and lead to death in every case. A brief

examination of Text-fig. 2 and Table II will show nevertheless that it is not until cultivation has proceeded for from 20 to 25 hours that much immediate clearing of the blood stream occurs. Even the cocci of a 16 hour and in some cases those of a 20 hour broth culture are for the greatest part still in a condition to resist the mechanism of the host,

Rabbits with Strain CH Cocci
Pneumococcus Type III per Cubic Centimeter of Blood

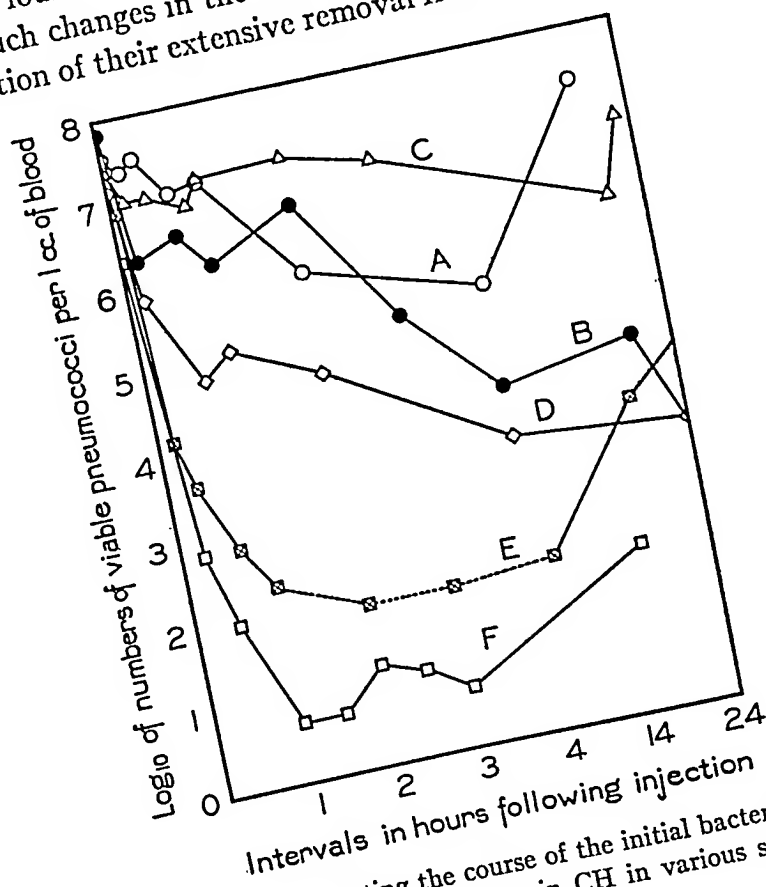
Force of inoculum

From blood of affected rabbit (No. 17, curve B)	Dextrose-serum broth cultures of varying ages				
	Rabbit 2-76, 3 hr. culture (Text-fig. 1, curve C)	Rabbit 2-78 3½ hr. culture	Rabbit 14, 4½ hr. culture (Text-fig. 1, curve D)	Rabbit 9, 5 hr. culture (Text-fig. 1, curve E)	Rabbit 1, 10 hr. culture (Text-fig. 1, curve F)
10 min.)	2.1 × 10 ⁷ (4 min.) — 9.2 × 10 ⁶ (15 min.) 3.1 × 10 ⁶ (30 min.)	2.6 × 10 ⁷ 1.1 × 10 ⁷ 1.1 × 10 ⁷ (25 min.) 7.2 × 10 ⁶ (40 min.)	1.5 × 10 ⁷ 7.8 × 10 ⁶ — 7.0 × 10 ⁴ (40 min.)	8.3 × 10 ⁶ (3 min.) 1.6 × 10 ⁴ 4.3 × 10 ³ 700 (40 min.)	2.3 × 10 ⁶ 680 (15 min.) — 90 (30 min.)
19 hrs.) 15 hrs.)	2.5 × 10 ⁵ 1.8 × 10 ⁵ 4.1 × 10 ⁵ — 2.9 × 10 ⁴ — 1.8 × 10 ⁴ (22 hrs.) — 2.4 × 10 ³ (46 hrs.) 1.7 × 10 ² (69 hrs.) — —	1.3 × 10 ⁷ 1.5 × 10 ⁷ 9.0 × 10 ⁶ — — 1 × 10 ⁶ (20 hrs.) 7.9 × 10 ⁵ (22 hrs.) — — — —	1.3 × 10 ⁵ 4.6 × 10 ⁴ — 3.0 × 10 ³ — — 2.0 × 10 ² (24 hrs.) — 150 (48 hrs.) — — —	200 <100 <100 — 100 (6 hrs.) 4.5 × 10 ³ (18 hrs.) — 2.3 × 10 ⁴ (26 hrs.) 3.0 × 10 ³ (42 hrs.) 300 (69 hrs.) 30 (92 hrs.) 2 (116 hrs.) 0 (136 hrs.)	5 5 (1½ hrs.) 15 (2 hrs.) 10 (2½ hrs.) 5 (3 hrs.) — 90 (16 hrs.) — — 22 (44 hrs.) — — —
	R	D in 26 hrs.	R	R	R

which is remarkably efficient in removing the organisms in a 25 or 30 hour culture.

If these results be compared with the data recorded in Paper I concerning the relative times at which capsular diminution and loss

begin, it will be found that there is in general a close agreement between the onset of such changes in the organisms as cultivation is prolonged and the initiation of their extensive removal from the blood. Because



TEXT-FIG. 1. Curves illustrating the course of the initial bacteremia following the intravenous injection into rabbits of strain CH in various states of encapsulation.

Curve A, organisms from mouse peritoneum.

" B, organisms in the blood of an infected rabbit.

" C, organisms from 3 hour broth culture.

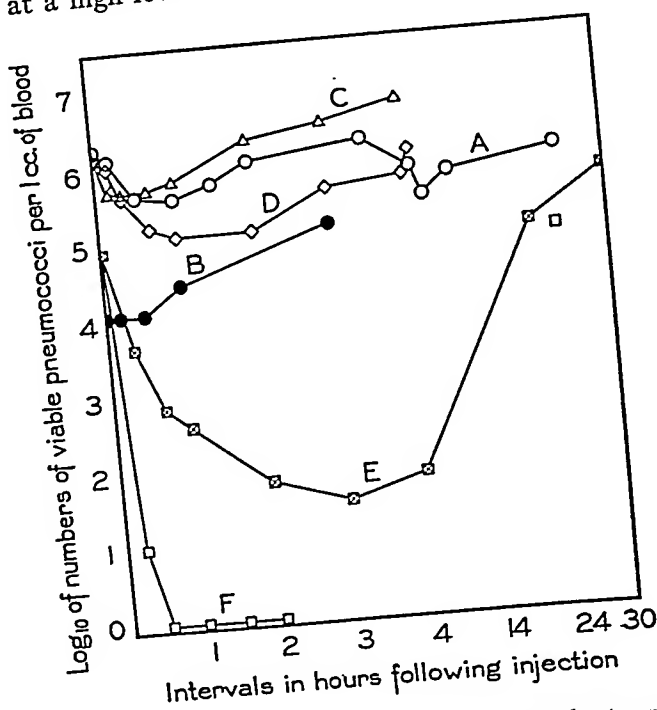
" D, organisms from 4½ hour broth culture.

" E, organisms from 5 hour broth culture.

" F, organisms from 10 hour broth culture.

of this correlation between capsular shrinkage and removal from the blood, we conclude that these phenomena are interdependent. The experiments have a further importance in our study since they demonstrate the fact that if the rabbit avirulent strain is injected when the capsule is intact, such as occurs in the case of "animal" organisms

or those in a 3 hour culture, it may not infrequently lead to the death of the animal, providing the number of organisms in the blood can be maintained at a high level—an outcome never observed when older,



TEXT-FIG. 2. Curves illustrating the course of the initial bacteremia following the intravenous injection into rabbits of strain SV in various states of encapsulation.

- Curve A, organisms from mouse peritoneum.
 " B, organisms in the blood of an infected rabbit.
 " C, organisms from 6 hour broth culture.
 " D, organisms from 16 hour broth culture.
 " E, organisms from 25 hour broth culture.
 " F, organisms from 30 hour broth culture.

partially decapsulated cocci, even in very large doses, have been used. We have noted, however, that the organisms observed in smears of the blood taken during the course of the bacteremia or postmortem possess much smaller capsules in the case of CH than those of SV.

Particular significance must be assigned to the persistence in the

blood of 16 hour cultures of the rabbit virulent strain, since Wright (4) noting the failure of young cultures of Pneumococcus Type I to disappear from the blood stream, concluded that this depended upon their existing in the logarithmic phase of growth. It is apparent from the growth curves presented in Text-fig. 1 of the first paper that, after 16 hours, strain SV has been in the stationary period for about 6 to 8 hours. Its resistance to removal cannot therefore be associated with

7A.

Intravenous Inoculation of S
Results of Blood Cultures (Expressed as Numbers of)

Time interval elapsing between infection and blood culture	Organisms from mouse peritoneum	Infected blood transferred from rabbit 5-04	Rabbit 5-03 5 hr. culture	Rabbit 4-97 5½ hr. culture
	Rabbit 5-04 (Text-fig. 2, curve A)	Rabbit 5-05 (Text-fig. 2, curve B)		
<i>min.</i>				
1	1.8×10^6	1.2×10^4	4.8×10^5	2.1×10^5
10	1.3×10^6	1.2×10^4	4.6×10^5 (6 min.)	1.6×10^5
20	—	—	3.2×10^5 (12 min.)	1.3×10^5 (25 min.)
30-40	4.2×10^5 (30 min.)	1.2×10^4 (30 min.)	4.5×10^5 (30 min.)	9.0×10^4 (40 min.)
<i>hrs.</i>				
1	3.7×10^5	2.8×10^4	8.3×10^5	1.6×10^5
2	5.6×10^5 (1½ hrs.)	—	1.2×10^6 (2 hrs.)	2.9×10^5 (2 hrs.)
3	6.0×10^5 (2 hrs.)	1.3×10^5	2.0×10^6 (2½ hrs.)	5.4×10^5 (2½ hrs.)
4	1.5×10^6 (3½ hrs.)	—	—	7.3×10^5
5	—	—	—	—
6	6.1×10^5	—	—	—
10-20	2.6×10^5 (6½ hrs.)	1.6×10^9 (16 hrs.)	—	—
21-26	4.3×10^5 (10 hrs.)	—	—	—
27-40	—	—	—	—
41-70	—	—	—	—
71 hrs. onward	—	—	D in 22 hrs.	D in 17 hrs.
Final outcome.....	D in 24 hrs.	D in 15¾ hrs.		

the logarithmic phase of growth, but rather with the presence of capsule. We wish to point out that a lag period is observed following the introduction into the animal of organisms in any condition of growth, which we consider to be induced by alteration of environment. The duration of this lag *in vivo* is, however, shortest when the organisms in the phase of increase in the blood of one animal are transferred to another (Text-

fig. 2, curve B), since probably, under these circumstances, the change in environment is minimal.

Difference in Virulence of Strains SV and CH in Mice Following Intravenous Injection

The differences revealed between the rabbit virulent strain SV and the rabbit avirulent strain CH by the studies reported in Paper II,

*bits with Strain SV Cocci
umococcus Type III per Cubic Centimeter of Blood)*

of inoculum

Dextrose-serum-broth cultures of varying ages

hr. culture (curve C)	Rabbit 4-02, 16 hr. culture (Text-fig. 2, curve D)	Rabbit 18 20 hr. culture	Rabbit 31 21 hr. culture	Rabbit 32, 25 hr. culture (Text-fig. 2, curve E)	Rabbit 23, 30 hr. culture (Text-fig. 2, curve F)
(40 min.)	1.3×10^6 1.1×10^6 4.2×10^5 1.6×10^5 (40 min.) 1.2×10^5 1.2×10^5 3.8×10^5 4.9×10^5 1.0×10^6 1.1×10^6 	1.9×10^5 1.0×10^5 8.7×10^5 6.6×10^5 (40 min.) 4.0×10^5 1.8×10^5 3.6×10^5 4.8×10^5 1.6×10^6 2.2×10^6 	1.0×10^6 (2 min.) 4.6×10^4 5.2×10^3 850 (40 min.) 240 0 0 0 — 	8.9×10^4 — 4.3×10^3 670 (40 min.) 360 60 30 60 — 	8.5×10^4 10 (15 min.) — 0 (30 min.) 0 0 —
hrs.	D in <24 hrs.	D in <18 hrs.	D in <66 hrs.	D in 48 hrs.	D in 117 hrs.

together with the experiments just recorded, made it seem improbable that their contrasting behavior in the rabbit was mainly attributable to any specific factor of the host. Since in the majority of Tillett's experiments and in our own, cultures were inoculated intravenously into rabbits, it seemed not unlikely that if the same route were adopted in the case of mice, variation in virulence might be revealed.

IMMUNITY TO PNEUMOCOCCUS TYPE III. III

Accordingly, a number of series of white mice were injected into one of the caudal veins with 0.2 cc. of 6 hour dextrose serum broth cultures of each strain appropriately diluted in sterile broth. Counts of the viable organisms present in each culture were obtained by duplicate platings of 0.1 cc. of the higher dilutions injected. In Table III are summarized the combined results of several titrations made at different times. Although the numbers of mice employed are doubtless too few to give the results statistical validity, nevertheless, we consider that the virulence of strain SV for these animals is probably about 10,000 times that of strain CH. Data obtained using the intraperitoneal route indicate that there is a possible three- to fourfold difference in virulence when this method of inoculation is adopted.

These experiments suggest that, even in the highly susceptible mouse, the rabbit avirulent strain, when introduced in a manner permitting

TABLE III
Titration of Virulence of Strains SV and CH in Mice Following Intravenous Inoculation

Strain	Nos. of pneumococci intravenously inoculated									
	10 ⁰ to 9 × 10 ⁰		10 ¹ to 9.9 × 10 ¹		10 ² to 9.9 × 10 ²		10 ³ to 9.9 × 10 ³		10 ⁴ to 9.9 × 10 ⁴	
	S	D	S	D	S	D	S	D	S	D
SV.....	1	0	7	9	2	14	1	15	nd	nd
CH.....	nd	nd	nd	nd	nd	nd	6	0	4	1
									6	6
									2	9
									1	16

S = survived for 1 week or longer.
D = died.

nd = not done.

Figures under letters indicate numbers of mice.

the normal defensive mechanism of the body to function effectively, is definitely less virulent than the rabbit virulent strain. The former, however, may cause the death of both rabbits and mice, if the dose is sufficiently large, and in the case of rabbits, at least, if injected when completely capsulated.

Having established the facts that disappearance from the rabbit's blood stream of both strains went *pari passu* with capsular loss, and that difference in virulence appeared to be related to differences in the organisms themselves, and not to the particular species employed for testing this property, we proceeded to investigate, by means of

various techniques, the mechanisms by which removal from the blood stream is accomplished, again with the purpose of demonstrating differences which might be related to the changes in the surface properties of the organisms which have previously been described.

Phagocytosis by Fixed Tissue Cells

Since it is well known that after intravenous injection followed by the removal of organisms from the blood they accumulate in the sinusoids of the liver, in the splenic pulp, the capillaries of the lung and even in the muscles, we were led to an examination of stained sections of certain of these tissues from normal animals which had received large intravenous injections of the two strains, CH and SV. In general, the results obtained in a number of animals thus prepared corroborated those of the two experiments, the details of which are presented below.

Two rabbits, weighing 2.59 and 2.64 kilos, were injected with the centrifuged deposits from 45 cc. and 30 cc. of 15 hour blood broth cultures of strains SV and CH, respectively. Blood cultures were taken after $\frac{1}{2}$ minute and 10 minutes. The organisms in the blood of the animal receiving strain CH were reduced from 1.38×10^8 per cc. to 3.72×10^4 per cc., while in the case of strain SV the counts were 3×10^8 per cc. after $\frac{1}{2}$ minute, and 1×10^8 per cc. after 10 minutes. Immediately after the last blood culture, the animals were killed by fracture of the cervical vertebrae, and portions of the organs fixed in Zenker's solution. From these stained sections were prepared.

Study of the sections revealed in the liver of the rabbit inoculated with strain SV moderate numbers of organisms, the majority of which were extracellular, lying free in the sinusoids. They appeared to possess a well defined capsule. A few cocci were seen located within the Kupffer cells, apparently having no capsules. In the spleen of this animal, again, only moderate numbers of organisms were observed. It was difficult to be certain in all cases as to their relation to the cells, but in many instances they were lying free in the blood spaces. Capsulation was noted. After prolonged examination of the lung, only one extracellular short chain of cocci was seen. In the bone marrow likewise only one chain was observed, lying free in the lumen of a blood vessel.

In another experiment, 28 hour broth cultures of SV were injected into three rabbits which were killed after 10 minutes, $2\frac{1}{2}$ hours and 4 hours. Unlike those in a 15 hour culture, most of the organisms injected were quickly removed from the blood stream of each animal. Examination of sections of the organs showed pneumococci in the Kupffer cells of the liver of the rabbit killed after 10 minutes and some in the phagocytic cells of the spleen. No extracellular cocci were

observed. In these organs from the animals killed after 2½ and 4 hours, practically no organisms—either intra- or extracellular—were seen.

The liver of the rabbit injected with strain CH revealed in certain areas large numbers of noncapsulated cocci, all of which appeared to lie within the Kupffer cells. As many as eight to nine cocci were counted within one Kupffer cell. Certain organisms stained poorly, contrasting with the intense coloration of others, suggesting in the former the onset of degenerative changes. In an occasional instance, it appeared that organisms had been taken up by polymorphonuclear leucocytes which themselves had been ingested by the Kupffer cells. Fewer cocci were observed in the spleen of this animal than in that of the rabbit injected with strain SV. Again, it was difficult to decide in every case concerning the intra- or extracellular position of the cocci, but some of them could be seen definitely within cells. Examination of the lung and bone marrow revealed in each only two diplococci within a polymorphonuclear leucocyte.

Upon examination of the organs of animals sacrificed after 7 hours or longer, in some cases only a very few and in others no organisms were seen.

From these and additional experiments of the same kind, it would appear that the majority of the organisms of strain SV, possessing as they do a capsule in a 15 hour culture, remained invulnerable to phagocytic action of the fixed tissue cells, particularly those of the liver. A minority which presumably had lost their capsules were taken up by such cells. The organisms from 28 hour cultures, many of which have lost their capsules, are rapidly ingested. The active phagocytosis of 15 hour cultures of strain CH, especially by the Kupffer cells of the liver, contrasts sharply with the behavior of strain SV at this age. Since it has been shown that in such CH cultures practically all of the capsule has been lost, we are again inclined to associate their susceptibility to phagocytosis by these cells as directly dependent upon the loss of this structure. In addition, the study of the sections suggests that the clearing of the blood is mainly effected by the fixed tissue phagocytes, although a certain number of organisms may be engulfed by polymorphonuclear leucocytes.

Fate of Organisms after Ingestion by Fixed Tissue Cells

It has thus been shown that the two strains disappear from the blood and are taken up by fixed tissue cells to an equal extent, provided the state of the organisms as regards lack of capsular material is the same, a condition obtained only with SV cultures of greater age. In the experiments under consideration here, we have sought to

determine whether ingestion by these cells leads to the destruction of both strains, or whether in one the bacteria may be killed and in the other survive and increase within these cells.

Counts were made of the viable bacteria in suspensions prepared from weighed portions of the organs from a series of rabbits killed at various intervals ranging from 10 minutes to 8 hours after intravenous infection. The counts were compared with those obtained from blood removed immediately before death. Rabbits were injected with the centrifuged suspensions from 30 to 40 cc. of blood broth cultures of the two strains taken up in a small quantity of sterile broth. 12½ to 15 hour cultures of strain CH and 15 hour and 28 hour cultures of strain SV were employed. In each case, the blood was cultured within ½ to 2 minutes following inoculation and again just before the animals were killed by a sharp blow over the occiput.

From the data obtained in representative experiments assembled in Table IV, it is evident that when there is only a slight diminution in the numbers of organisms in the circulating blood during the interval between injection and death, the ratio obtained by dividing the bacterial count for 1 gm. of liver, spleen or lung by that for 1 cc. of blood drawn immediately before the animal was killed is small, indicating that the organisms cultured from the organ sample are chiefly in the contained blood. This is exemplified by the rabbit injected with 15 hour SV organisms and killed within 10 minutes. When, however, a large decrease in the number of organisms occurs in the blood, as is apparent with the animals inoculated with a 28 hour culture of SV or a 15 hour culture of CH, and killed after 10 minutes, this ratio markedly increases, reflecting the accumulation of bacteria in the phagocytic cells of these organs. If the time of death be postponed for from 2 to 4 hours, the ratios obtained with 13 hour CH and 28 hour SV cultures again diminish and are roughly of the same magnitudes as those found for 15 hour SV. We believe therefore that in these animals killed from 2 to 4 hours after injection, which show low ratios, the organisms cultivated from the organs in greater part at least were present in the contained blood, and that those which had been taken up by the fixed tissue phagocytes were destroyed during this period.

Additional evidence for this view is found in the results of similar experiments, not presented in detail, in which ratios were recorded for 13 hour CH, when the animals were sacrificed after 40 minutes and 1 hour subsequent to injection, that

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are intermediate between those for the rabbits killed after 10 minutes and 2 hours. Thus, the 40 minute and 1 hour ratios for liver to blood were 120 and 12 respectively. It will be seen that the ratio for 28 hour SV is still large at the end of 2½ hours, whereas before this time that of 13 hour CH has fallen to a minimum. The explanation probably lies in the fact that the 28 hour SV culture employed was removed from the blood somewhat more slowly than the 13 hour CH. Thus,

TABLE IV
Relative Numbers of Pneumococcus Type III in Tissues and in Blood at Varying Intervals after Intravenous Inoculation

Material cultured	Rabbit 1 killed after 10 min. Strain SV (15 hrs.)		Rabbit 2 killed after 10 min. Strain CH (15 hrs.)		Rabbit 3 killed after 1 hr. 50 min. Strain CH (13 hrs.)		Rabbit 4 killed after 8 hrs. Strain CH (13 hrs.)	
	Log. No. Pn*	Ratio†	Log. No. Pn	Ratio	Log. No. Pn	Ratio	Log. No. Pn	Ratio
Blood immediately after inoculation	7.917	—	8.140	—	9.328	—	9.226	—
Blood before death	7.586	—	4.571	—	5.306	—	5.602	—
Liver	6.594	0.1	7.328	570.0	4.462	0.15	4.538	0.098
Spleen	7.422	0.67	5.980	26.0	5.803	3.2	6.305	5.0
Lung	6.288	0.05	5.154	3.0	3.000	0.005	4.516	0.082
Material cultured	Rabbit 5 killed after 8 hrs. Strain CH (13 hrs.)		Rabbit 6 killed after 10 min. Strain SV (28 hrs.)		Rabbit 7 killed after 2 hrs. 10 min. Strain SV (28 hrs.)		Rabbit 8 killed after 4 hrs. Strain SV (28 hrs.)	
	Log. No. Pn	Ratio	Log. No. Pn	Ratio	Log. No. Pn	Ratio	Log. No. Pn	Ratio
Blood immediately after inoculation	8.493	—	7.256	—	6.977	—	5.414†	—
Blood before death	4.105	—	4.681	—	2.114	—	2.684	—
Liver	3.398	0.25	5.987	20.2	3.881	60.0	3.042	2.38
Spleen	4.267	1.9	6.021	21.9	4.820	507.0	3.279	3.94
Lung	3.000	0.1	3.903	0.167	2.699	3.85	2.000	0.21

* Log. No. Pn = logarithm of the number of pneumococci per 1 gm. of tissue or 1 cc. of blood.

† Ratio = $\frac{\text{Number of pneumococci per 1 gm. of tissue}}{\text{Number of pneumococci per 1 cc. of blood}}$

‡ Blood taken after 3 minutes.

the ratio increases up to 2 hours after injection, denoting the storage of still viable cocci in the organs. By the end of 4 hours, however, these values have dropped to the low figures approximating those earlier found for CH which, as already noted, we consider to be indicative of the destruction of most of the cocci.

From these results we may conclude that when the organisms of both rabbit virulent and avirulent strains are taken up by the phagocytic

cells of certain organs, the majority are thereafter destroyed within about 2 hours. There is no evidence indicating any capacity on the part of the rabbit virulent strain to resist the bactericidal action of these cells once the integrity of the capsule has been impaired and an intracellular position has been assumed.

Phagocytosis by Polymorphonuclear Leucocytes

Tillett, in his studies on the immunity of rabbits to smooth Type III pneumococcus, failed to find evidence for the phagocytosis of this organism by polymorphonuclear leucocytes, whether or not it was virulent for these animals. He employed a method involving the use

TABLE V
Phagocytosis of CH and SV of Varying Age by the Normal Polymorphonuclear Cells and Serum of Rabbits and Mice

Source of cells and serum	Strain	Age of culture	Average No. of cocci per 10 cells	Cells containing organisms	No. cells counted
		<i>hrs.</i>		<i>per cent</i>	
Rabbit A	CH	6	5.1	9.0	200
	SV	6	0.04	0.02	500
	CH	16	87.0	86.0	50
	SV	16	13.7	30.0	100
Mouse A	CH	22	69.0	81.0	300
Mouse B	CH	22	11.2	33.0	262
Mouse C	CH	22	30.0	55.0	250

of a vital stain and direct observation of mixtures of serum, leucocytes and bacteria on a warm stage. His failures, together with our observations noted above of occasional cocci in this type of leucocytes in sections of tissues, prompted a further investigation of this phenomenon.

Suspensions of strains CH and SV from dextrose serum broth cultures of varying ages were added to mixtures of the exudative leucocytes and the defibrinated blood of normal rabbits, and rotated in sealed tubes at 38.5°C. Smears of the contents were made after $\frac{1}{2}$ hour and counts of the organisms within polymorphonuclear leucocytes carried out. These, summarized in Table V, clearly demonstrate a considerable ability on the part of the rabbit leucocytes to ingest both strains, provided cultures of a sufficient age are employed. As was found when they were exposed to the action of human defibrinated blood (*cf.* Paper II),

there is a disparity between the time at which the organisms of the two strains become susceptible to the attack by the leucocytes. Thus, about seventeen times as many CH organisms were taken up by leucocytes when derived from a 16 hour broth culture as when the culture is 6 hours. Even in the latter, there is definite phagocytosis, however, whereas with a 6 hour culture of strain SV the numbers found in leucocytes are practically negligible. After 16 hours cultivation of this strain, however, a certain amount of phagocytosis is observed.

It was also possible to demonstrate *in vitro* considerable activity of the polymorphonuclear leucocytes of the mouse against older cultures. In one experiment, the details of which are also recorded in Table V, the exudative leucocytes from the peritonea of three mice, mixed with the homologous defibrinated blood, exhibited definite ability to take up a suspension of CH organisms from a 22 hour broth culture. It is interesting to note, in view of the well known variation in the susceptibility of normal mice to pneumococcus, that the numbers of organisms phagocytized by the cells and serum of the individual mouse are significantly different in each case. 10 hour cultures of this pneumococcus strain were definitely more resistant to phagocytic action although a small number were seen within cells.

These experiments then make it clear that the cells and serum of the normal rabbit and even the very susceptible mouse can bring about the ingestion of virulent *Pneumococcus* Type III. One of the factors which determines the extent to which this is accomplished is the degree of decapsulation of the cocci.

Phagocytosis by Exudative Leucocytes in Vivo

Since it was definitely established that polymorphonuclear leucocytes of the rabbit and mouse could, in the test tube, ingest large numbers of *Pneumococcus* Type III, but that this depended upon the state of the culture employed, we proceeded to determine whether or not these cells when present in an inflammatory exudate within the body behaved in the same manner. For this purpose an exudation of cells was produced in the peritoneal cavities of mice, and in the pleural cavities of rabbits by injections of sterile saline or broth as described in the section on technique.

1. *In the Mouse*.—Studies of smears prepared from the peritoneal exudate of normal mice removed 30 minutes after injection of suspensions of organisms of various ages obtained from broth cultures have shown that, in general, the results secured *in vitro* using mixtures of cells and serum were reproduced in the living susceptible animal. From the data presented in Table VI, it is apparent that if the culture inoculated be sufficiently old, the polymorphonuclear leucocytes of

the mouse are capable of taking up considerable numbers of both the rabbit virulent and avirulent organisms. Young cultures of the latter are much more resistant to phagocytosis, although it is evident that a small proportion of even $4\frac{1}{2}$ hour culture of this strain may be ingested, while $4\frac{1}{2}$ and 6 hour cultures of strain SV appear to be completely resistant. After a growth period of 12 hours, strain CH exhibits marked susceptibility to phagocytic attack, contrasting in this respect with the behavior of SV organisms of the same age, some of which, nevertheless, have now become definitely vulnerable. Although in certain instances, cocci were observed within large mononuclear cells, the majority were found in polymorphonuclear leucocytes.

These experiments serve to emphasize the importance of the relationship between the conditions at the surface of the organism at the

TABLE VI

Phagocytosis of Strains CH and SV by Polymorphonuclear Leucocytes in the Peritoneal Cavity of Mice

Strain	Age of culture	Average No. of cocci per 10 cells	Cells containing organisms	No. cells counted	Remarks
	<i>hrs.</i>		<i>per cent</i>		
CH	$4\frac{1}{2}$	0.8	2	50	Further examination of preparation showed no phagocytosis
SV	$4\frac{1}{2}$	0.0	0	50	
CH	6	2.25	8	40	Capsules on most extracellular cocci
SV	6	0.0	0	50	Capsules on most extracellular cocci
CH	12	92.0	80	25	Some cocci in large mononuclears—majority in polymorphs. No capsules on majority of extracellular cocci
SV	12	11.0	26	50	Phagocytosis observed only in polymorphs. Capsules on most extracellular cocci

time it is introduced into the body and the capacity of the local defensive mechanism of the host to attack it.

2. *In the Rabbit.*—Singer and Adler (5), in their studies on the immunity of the rabbit against *Pneumococcus* Type III, failed to observe any phagocytosis by polymorphonuclear leucocytes in pleural exudates. These authors, noting the presence of pneumococci within endothelial cells not only in the pleural cavity but in bone marrow and elsewhere, concluded that their elimination from the rabbit was exclusively dependent upon the phagocytic properties of the cells of the reticulo-endothelial system. Since there appeared to be a discrepancy between their findings and our demonstration of definite phagocytosis of both strains CH and SV by rabbit polymorphonuclears *in vitro* and by analogous cells in the peritoneum of the mouse, it became of interest to repeat the experiments of Singer

and Adler, using the two strains which have been studied. Furthermore, we wished to follow locally the course of events in the pleural exudate of rabbits until death or recovery occurred, with the object of correlating the state of the pneumococcus in respect to changes in the capsule with the degree of phagocytic activity should this be shown to occur.

In Table VII are summarized the observations made in the cases of two rabbits prepared by preliminary intrapleural injection of broth. One was inoculated with a 23 hour culture of strain CH and the other with a SV culture of the same age. An examination of the data will show that within 17 minutes after the injection of organisms in this late stage of growth, active phagocytosis took place in which both polymorphonuclear and mononuclear cells shared. This process was sufficiently effective to lead, within an hour, to a marked reduction in the numbers of extracellular cocci, which was reflected, at least in the case of strain CH, by about a ninefold reduction in the count of viable organisms present in the exudate. This fact, together with the striking decrease in the numbers of intracellular cocci of both strains observed after 1 hour, affords a certain amount of evidence for believing that the large proportion of the organisms originally introduced, which were seen within cells after 17 minutes, were rapidly killed and digested. We found no evidence of intracellular multiplication corresponding to that of Goodner and Miller (6), who believe that in the peritoneum of the normal mouse the pneumococci (Type I) ingested by the leucocytes multiply within the cell, ultimately causing it to rupture and in this manner are again liberated. By the end of 2 hours little change occurred in the exudate containing strain CH except that there was some indication of beginning encapsulation without any increase in the number of viable organisms. On the other hand, a definite multiplication of strain SV had begun and the cocci exhibited well developed capsules. The capsules were numbers of capsulated organisms were seen in both exudates. This proportion of susceptible CH organisms was greatly enlarged by 21½ hours, although strain CH had again become susceptible to ingestion by leucocytes. This proportion of susceptible CH organisms was greatly enlarged by 21½ hours, although there were still many extracellularly situated. In the case of SV there was little evidence of phagocytosis at this time. Thereafter, until the death of the animal at 47½ hours, the extracellular encapsulated cocci were numerous and only a very few were seen within leucocytes. During the period of 49 hours following the onset of the marked phagocytosis of strain CH observed at 21½ hours, the numbers of viable organisms, which had been significantly reduced by 45 hours, remained more or less constant. Throughout this interval there was indication of great activity on the part of the leucocytes in taking up the organisms and, judging by the appearance of the intracellular cocci, in destroying them. Subsequently, the number of organisms within cells diminished, but complete sterilization of the exudate was not obtained until 94 hours later. In another similar experiment involving strain CH, no viable organisms were found in the exudate over a period extending from 44 hours to 90 hours after injection. At 120 hours, great numbers of encapsulated pneumococci were seen. 20 hours later the exudate became

TABLE VII

Phagocytosis of Pneumococcus III within the Pleural Cavity of the Rabbit

Time after infection at which exudate withdrawn	Strain CH		Strain SV	
	Remarks on microscopic examination of stained exudate	No. organisms per 1 cc. of exudate	Remarks on microscopic examination of stained exudate	No. organisms per 1 cc. of exudate
<i>min.</i>				
17	Marked phagocytosis by polymorphs and mononuclears. Moderate numbers of extracellular cocci	3×10^6	Considerable phagocytosis—more by mononuclears than by polymorphs but definite in latter. Small numbers extracellular noncapsulated cocci	Confluent growth from 0.005 cc.
<i>hrs.</i>				
1	Only 2 polymorphs and 2 mononuclears seen with ingested cocci. Very few pneumococci seen extracellularly	4×10^5	Only a few organisms seen outside cells; none within. Evidence of beginning encapsulation	Confluent growth from 0.005 cc.
2	Practically no evidence of phagocytosis. Extracellular cocci infrequent. Those seen had small capsules	2×10^5	No phagocytosis noted. Marked increase in number of extracellular organisms which have large capsules	1×10^5
7½	A few cocci in polymorphs and mononuclears. Many extracellular encapsulated cocci in short chains	Confluent growth on plating 0.01 cc. of exudate	No phagocytosis. Large numbers of extracellular cocci in chains having huge capsules	Confluent growth from 0.02 cc.
21½	Marked phagocytosis by polymorphs. Extracellular organisms numerous	9×10^5	A few cocci in mononuclears; none in polymorphs. Moderate numbers extracellular capsulated cocci seen	1.3×10^5
27	Failed to obtain exudate		No phagocytosis noted. Numerous extracellular encapsulated organisms	Confluent growth from 0.001 cc.
45	Very marked phagocytosis by polymorphs and monocytes. Intracellular organisms stain best by Gram and poorly by Wright. Indication of beginning digestion? Very few extracellular cocci	3.5×10^3	Some phagocytosis by mononuclears and to a lesser degree by polymorphs. Numerous extracellular encapsulated organisms	1.5×10^5
47½	Extensive phagocytosis; about 30 to 40 per cent of polymorphs contain cocci. As many as 10 to 20 may be seen in one cell. Gram stain better than Wright. Very few extracellular cocci	1×10^4	No definite phagocytosis. Large numbers of heavily capsulated, extracellular organisms. Animal died at this time	Confluent growth from 0.001 cc.
70½	Less phagocytosis than at 47½ hours. Cocci within cells appear to be disintegrated. No organisms seen extracellularly	1×10^5		
93½	No phagocytosis by polymorphs. A few cocci in mononuclears. A few extracellular capsulated cocci; one chain in about 10 fields	Cultures contaminated		
140½	No phagocytosis. No extracellular organisms seen	9×10^3		
164	No phagocytosis. No extracellular organisms seen	0		

sterile, but smears stained by Gram revealed many intracellular, partially digested cocci.

We wish to point out that the changes in the bacterial population in the exudates parallel fairly closely the course of the bacteremia in animals intravenously inoculated with the two strains, as will be seen from the data recorded in Tables I and II.

In summary, it may be stated that the ultimate fate of these two strains in the pleural exudates of rabbits appears to depend upon the difference in their ability to maintain a resistance to ingestion by leucocytes after the initial decline and subsequent phase of increase has occurred. We consider that this difference in the organisms which have developed within the body is associated with the same properties that have been shown to account for their resistance or susceptibility to phagocytosis, when they have been cultivated in artificial media. It is not improbable that with strain CH growing in the exudate a balance is soon struck between the rate of production of young invulnerable forms and the rate at which changes take place at the surfaces of the cocci, which render them susceptible. In this manner the number of extracellular viable organisms is restricted and in some cases may be reduced almost to zero. Since in strain SV these changes proceed more slowly, no such balance is established within the short time that these animals survive. The numbers of resistant forms quickly come to enormously outnumber those which have lost most of their capsule and in consequence have been removed by leucocytes.

DISCUSSION

Up to the present no adequate explanation has been offered for the observed differences in the virulence for rabbits of various strains of *Pneumococcus* Type III. In Paper I of this series it was shown that smooth strains of the organism could be grouped on the basis of their ability to remain viable and to multiply at 41°C. or their lack of this quality. The majority of strains tested died off more or less rapidly at this temperature which is usually attained within a few hours after intravenous infection by normal rabbits. Such strains, when tested for rabbit virulence by this route, failed to kill. All of the virulent strains grew at 41°C. but some of the "thermo-resistant" strains were avirulent in the quantities injected. It appeared that the ability to

proliferate at 41°C. was a prerequisite but not the sole factor in determining the degree of virulence of a given strain. In an attempt to further analyze the problem we selected for study two strains which differ in their lethal properties for rabbits, but both of which are able to grow at 41°C. In Paper II we have determined that *in vitro* at 37°C. the avirulent strain CH produces a somewhat smaller capsule than the rabbit virulent strain SV and, furthermore, that the loss of the structure during cultivation which leads to surface changes giving the cells, at least temporarily, properties similar to those of the R organisms, occurs more rapidly in the former. In this paper it has been demonstrated that the extent of capsular disintegration at the time of injection is directly correlated with the capacity of the animal body to remove both strains from the blood and to eliminate them through ingestion and destruction by means of the phagocytic cells, chiefly those of the fixed tissues but to a certain degree by the mobile leucocytes as well. Furthermore, from an examination of the events transpiring within the pleural cavity subsequent to the introduction of the two strains, it appears that just as *in vitro*, so in the body, diminution of the smaller CH capsule (which at first speedily develops) takes place readily and advances swiftly, leading to extensive phagocytosis by polymorphonuclears. In contrast, the vast majority of SV cocci, after the production of their massive capsules, remain invulnerable in the presence of leucocytes during the period preceding the death of the animal.

In view of all the foregoing experimental data we feel justified in asserting that (a) the inherent physiological potentiality of the bacterium to grow in the body of the host under the environmental conditions, including elevated temperature, encountered following infection, (b) together with the ability of the organism to maintain the capsule intact for a longer or shorter period of time, are fundamental factors in determining whether the injection of a given strain of smooth Type III pneumococcus will ultimately result in an invasion of the host characterized by practically unlimited proliferation of the organisms and ending in death, or whether the disease will be marked by a restrained increase in the cocci reflected by a controlled, low grade, fluctuating bacteremia which is ultimately overcome.

There is no difficulty in perceiving the application of this statement

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In view of all the foregoing experimental data we feel justified in asserting that (a) the inherent physiological potentiality of the bacterium to grow in the body of the host under the environmental conditions, including elevated temperature, encountered following infection, (b) together with the ability of the organism to maintain the capsule intact for a longer or shorter period of time, are fundamental factors in determining whether the injection of a given strain of smooth Type III pneumococcus will ultimately result in an invasion of the host characterized by practically unlimited proliferation of the organisms and ending in death, or whether the disease will be marked by a restrained increase in the cocci reflected by a controlled, low grade, fluctuating bacteremia which is ultimately overcome.

There is no difficulty in perceiving the application of this statement

to the case of the virulent strain SV. Given the ability to proliferate rapidly in the environment of the host, the production and retention of a large capsule insure it against destruction by phagocytes and permit multiplication to proceed practically unhindered. In considering the behavior of CH we will recall that this organism while able to grow at 41°C. also dies off much more quickly at such temperature. This fact together with the rapid loss of the capsule may serve to explain why the cocci found in the blood during the phase of secondary bacteremia, and which in order to remain there must be equipped with capsules, do not multiply unchecked and overwhelm the animal. Complete sterilization of the blood stream, which usually occurs on the 5th or 6th day after infection, is probably brought about by the development of antibodies which may be type specific (unpublished experiments) or species specific (cf. Paper IV¹).

The fundamental significance of temperature is further emphasized by the information presented here. The phagocytes of the rabbit are able to attack the virulent or avirulent Type III pneumococci, but only on the loss of their protective envelope. Since nearly all of the strains in our group 3 (cf. Paper I) which were susceptible to the deleterious effect of elevated temperatures possessed very large capsules, sometimes exceeding in size those of SV and retained *in vitro* for long periods, one might have anticipated that they would have remained nonphagocytizable by the cells of the host and therefore highly lethal. The lack of virulence of these strains with very large capsules can only be attributed to their failure to grow in the blood stream the hyperthermy developed and their removal from the blood stream following death. Strain Tirrell in group 2 (Paper I), which was also able to persist at 41°C., resembled CH in possessing capsules which (although slightly larger) were likewise speedily impaired, and its avirulence would appear to depend on this attribute. In contrast, strain IE with similar growth properties at 41°C. and possessing a very large persistent capsule proved highly virulent.

Although our study has been principally directed toward the analysis of the factors which account for the difference in virulence of Pneumococcus Type III strains in rabbits, we believe that these findings have a broader applicability since it has been shown that a similar distinction

¹ Enders, J. F., Wu, C.-J., and Shaffer, M. F., *J. Exp. Med.*, 1936, 64, in press.

in virulence for mice is exhibited provided the intravenous route be employed. This fact affords further evidence that the attributes which determine virulence are to be sought in the bacteria themselves, rather than in the particular species of host. We cannot, therefore, agree with the hypothesis of Tillett, that the rabbit possesses a host specific mechanism which enables it to destroy the capsule of the avirulent Type III *Pneumococcus*, but not that of the virulent variety.

The importance of capsular retention by the *Pneumococcus* as it affects the removal of the organisms from the blood has not been generally recognized by previous workers in this field. Thus, Teale (7) finds that the young actively growing cultures of virulent *Pneumococcus* Type III in contradistinction to nearly all other species of bacteria are not rapidly eliminated by the normal clearing mechanism of the blood. He definitely asserts that this "is not due to a capsule interfering with phagocytosis since it is not present in cultures," but presents no alternative explanation. Wright, observing the similar behavior of young cultures of Type I *Pneumococcus* following intravenous inoculation into rabbits attributed their persistence in the circulation to the fact that they were in the logarithmic growth phase, which continued unchecked after introduction into the animal. We do not feel, at least in the case of *Pneumococcus* Type III, that this is due to the phase of active growth *per se*, but rather to the fact that the size of the capsule is greatest at this stage. This view is borne out by certain experiments with SV in which the organisms, although long past the logarithmic growth period, still possessed large capsules and were not removed following injection. Further, the injection of CH cultures still in the phase of active multiplication was followed by a marked diminution in the numbers of cocci in the circulating blood. Finally, we have found that even when SV organisms, which had previously been increasing rapidly in the blood of one animal, are transferred to another, there is a short but definite initial period during which no increment in numbers is observed.

SUMMARY AND CONCLUSIONS

Among the experimental findings reported in this paper to which we wish to give particular emphasis are the following:

1. The results which follow the intravenous injection into rabbits

of two strains of Pneumococcus Type III of different degrees of virulence vary with the state of the capsule. Thus when this structure is completely developed both remain in the blood. A culture of either strain begins to become susceptible to the blood-clearing mechanism contemporaneously with the onset of capsular degeneration and the initiation of other concomitant changes at the surface of the organism (cf. Paper II), which occur much earlier with the less virulent strain.

2. When, in either case, removal from the blood stream occurs, this is effected by the phagocytic cells of the body. There is no suggestion that a new or unknown mechanism is involved. The greatest share of the burden is borne by the fixed phagocytic cells of the liver and spleen, and to a less extent by those of the lung and bone marrow. Nevertheless, it has been demonstrated that the polymorphonuclear leucocyte may also participate.

3. Phagocytosis by the leucocytes of the normal animal either *vitro* or *in vivo* has been observed only at such a time as the capsule has become impaired. Ingestion of the organisms by the fixed tissue cells appears also to be effective only under the same condition and is accordingly observed with much younger cultures of the less virulent strain.

4. Following their removal from the blood and their accumulation within the fixed phagocytes of the organs, destruction of most of the cocci proceeds within 2 to 4 hours. Both strains are destroyed provided they are in the state favorable to phagocytic attack.

5. Evidence has been presented which indicates that just as *in vitro*, so in a local area of inflammation within the body, aging with attendant capsular loss and increasing susceptibility to phagocytosis may take place.

6. With organisms from either strain a variable period of lag follows their injection into the blood stream, even when they are introduced in a state of active multiplication and complete encapsulation.

7. Differences in virulence for rabbits of two strains of Pneumococcus Type III do not imply that this animal possesses a defensive mechanism which is absent in other species, since it has been possible to demonstrate similar differences when the organisms are injected intravenously into mice. This fact indicates that the factors determining the degree of virulence of these strains are to be sought in the organisms themselves, rather than in the kind of host.

8. Differing degrees of virulence among various strains of *Pneumococcus* Type III capable of growing at the elevated temperature encountered in the infected animal appear to be conditioned by variations in the capacity to maintain the integrity of the capsule within the body.

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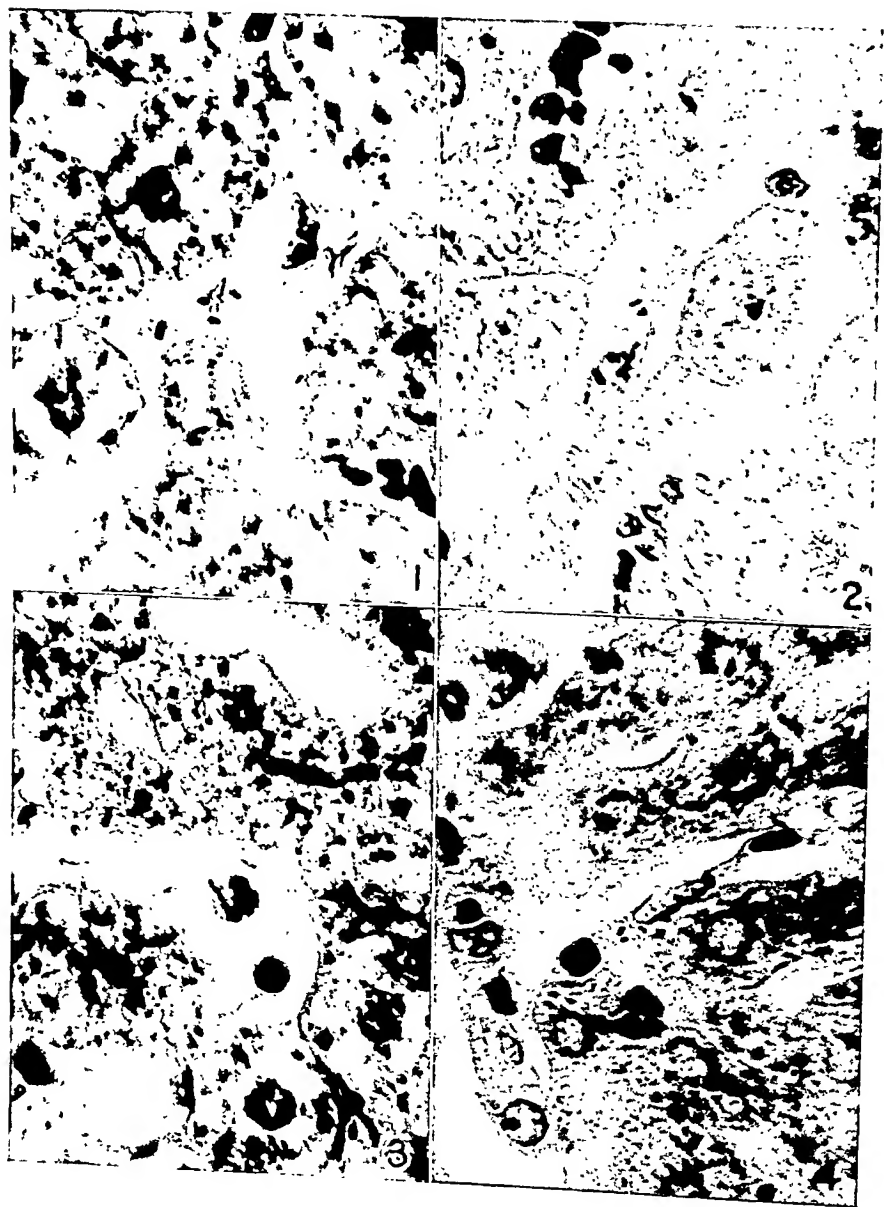
EXPLANATION OF PLATE 21

FIG. 1. Liver of rabbit injected with 15 hour broth culture of strain SV. Large encapsulated organisms lying free in sinusoid about a fragment of adventitious debris.

FIG. 2. Liver of same rabbit. Pneumococci in Kupffer cell; compare with Fig. 1, noting absence of capsule.

FIG. 3. Liver of same rabbit. Short chain of pneumococci apparently within a polymorphonuclear leucocyte lying in the sinusoidal space.

FIG. 4. Liver of rabbit injected with 15 hour broth culture of strain CH. Pneumococci in Kupffer cell. Note absence of capsule.



(Enders *et al.*: Immunity to Pneumococcus Type III. III)

AN UNSATURATED FATTY ACID FRACTION OF PIG PANCREAS WHICH INHIBITS THE GROWTH OF CHICKEN SARCOMA

By O. M. HELMER, Ph.D.

(From the Lilly Laboratories for Clinical Research, Indianapolis City Hospital, and the Lilly Research Laboratories, Indianapolis)

(Received for publication, June 3, 1936)

From time to time articles have appeared in the literature showing that the higher fatty acids exert an inhibiting action on tumor growth. Webb (1) in 1901 used soap solution in the treatment of human tumors. On the basis of Webb's work Shaw-Mackenzie (2) and Gardner (3) reported on the use of sodium oleate in the treatment of cancer. Nakahara (4) found that unsaturated fatty acids when injected intraperitoneally caused an increased resistance to the growth of subsequently implanted Bashford adenocarcinoma. Similarly, the growth of autografts of spontaneous tumors was retarded. Bierich (5) also reported an increased resistance to cancer implantation by means of an unsaturated fatty acid. Lecloux (6), in a paper in which he gave a comprehensive review of lipids in relation to cancer, reported that sodium oleate, oleic acid, and stearic acid when applied locally in intervals between painting retarded the appearance of tar cancer and that an iron salt of oleic acid exerted the same effect when injected into the peritoneum.

The growth of chicken tumors also is affected by the unsaturated fatty acids. Begg and Aitken (7) reported that intratumoral injections of sodium oleate sometimes led to regression and occasionally to complete disappearance of tumors and also that potent Rous filtrates were found to be inactivated by the addition of a neutralized solution of sodium oleate. Pirie (8), in studying the inhibiting action of pancreatic extracts on the Rous and Fujinami tumors, concluded that the inactivating factor of the pancreatic extracts was associated with the fatty acid and lecithin fractions. Baker and McIntosh (9) and Sugiura (10) also found that aqueous extracts of pancreas inhibited the chicken sarcoma, and Vassiliadis (11) mentioned that organo-extracts of pancreas had a retarding action on tar tumors.

It is the purpose of this paper to present a stepwise fractionation of fresh pancreatic tissue which demonstrates that the inhibiting action of this tissue against the Rous chicken sarcoma is found principally in the unsaturated fatty acid fraction.

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It is the purpose of this paper to present a stepwise fractionation of fresh pancreatic tissue which demonstrates that the inhibiting action of this tissue against the Rous chicken sarcoma is found principally in the unsaturated fatty acid fraction.

Materials and Methods

The chicken sarcoma used in these experiments was Chicken Tumor I, Rockefeller Institute series.¹ Frozen pig pancreas was used in all of the experiments because pig pancreas was found to be the richest source of the inhibiting agent. Moreover, it was also found to be unnecessary to dry the pancreas before extraction.

The chicken tumor extracts were prepared from desiccated tissue. 1 gm. of the powdered desiccate was extracted with 60 cc. of sterile distilled water. During the extraction the reaction was adjusted to pH 7.2-7.4 by the addition of 0.1 N NaOH. The debris was removed by centrifugation and the supernatant fluid filtered through coarse paper.

The extracted pancreatic lipids to be tested were dissolved in ether and a known amount was pipetted into a 15 cc. centrifuge tube. The ether was removed by immersing the tube in warm water and removing the last traces of ether by application of a vacuum. Then an appropriate amount of 1 per cent Na_2HPO_4 was added, the mouth of the tube closed with cotton, and the tube boiled in a water bath for 15 minutes to disperse the lipids and for sterilization. While the tube was still hot the cotton was removed and a sterile rubber cap substituted. The tube was then shaken so that the fatty substance would form a fairly stable emulsion. The tube was cooled to room temperature, an equal volume of the chicken tumor extract was added, and the mixture was allowed to stand at room temperature until injected.

A control tube was similarly prepared, using an equal amount of a solution of 1 per cent Na_2HPO_4 .

The experimental mixtures and their controls were injected intradermally into the breasts of young Plymouth Rock hens. In each case the amount injected was 0.4 cc. Six to eight injections were made into the breast of each hen. To eliminate differences in susceptibility of the chickens the measurements of the experimental tumors were recorded when the control tumors were of a fixed size. The inhibiting activity of the various fractions is expressed as the amount of inhibitor necessary to inactivate completely 0.2 cc. of the 1:60 chicken tumor extract.

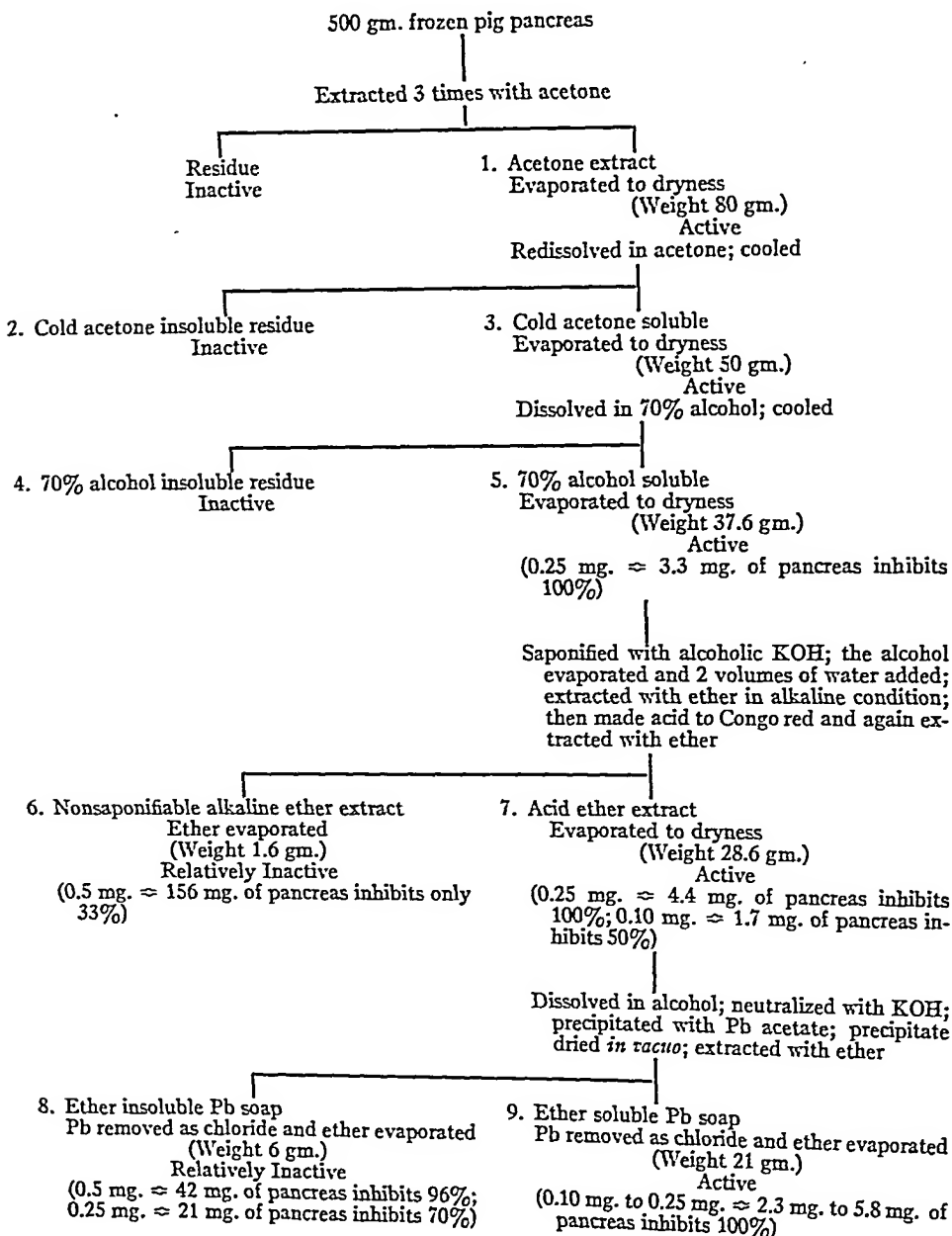
To obtain the data reported in this paper 825 injections were made into 127 chickens. For simplicity the results are presented in tabular form.

Fractionation.—Since frozen tissue was used as a starting material acetone and alcohol were tried as the preliminary extractives. Both proved satisfactory, but as acetone was preferable it was finally chosen for the first extraction. After removal of the acetone and water various solvents, such as benzene, ether, and acetone, were used in the second step of the fractionation. Acetone was again found to be the most suitable. The fractionation finally adopted is shown in Table I.

The unsaturated fatty acid fraction (9) had an iodine number of 89 and was an oil at room temperature. This fraction was also deeply pigmented, the pigment following the activity throughout the fractionation. It could not be further purified.

¹ This was kindly supplied by Dr. James B. Murphy.

TABLE I

Fractionation of Pancreatic Tissue for the Isolation of a Tumor-Inhibiting Agent

GROWTH-INHIBITING FRACTION OF PANCREAS

fied by precipitation from organic solvents. Therefore the following procedures, which are not shown in the table, were utilized.

First, the unsaturated fatty acid fraction (9) was distilled under the full vacuum of the cenco hyvac pump. A light yellow oil weighing 14 gm. distilled over at a pressure of about 1 mm. and a temperature of 176°C . This oil in quantities of 0.25 mg. inhibited 100 per cent. The tumor activity was inactivated also by 0.25 mg. of the dark brown residue. The distillate was liquid at room temperature and solidified in the ice box; it had an iodine number of 90, which corresponds with that of a fatty acid having one unsaturated group. When dissolved in alcohol and titrated with alcoholic KOH 0.3705 gm. and 0.4514 gm. required for neutralization 12.99 cc. and 15.57 cc., respectively, of 0.1 N KOH. These figures correspond to a molecular weight of 286.7 and 282.8 as compared to 282.36 for the molecular weight of oleic acid. Therefore it appears that the iodine number, the molecular weight, and the physical properties compare quite closely with those of oleic acid.

Commercial oleic acid, when tested in the same way as the pancreatic fractions, was found to have almost the same inhibiting power as the pancreatic fractions; 0.25 mg. of oleic acid inhibited 100 per cent, whereas 0.10 mg. of oleic acid did not cause complete inhibition.

In a second procedure the unsaturated fatty acid fraction was dissolved in ether and extracted with 5 per cent NaOH in a separatory funnel. The alkaline aqueous phase was then removed, acidified with HCl, and re-extracted with ether. This procedure was repeated twice and the final ether extract washed with water, of the pigment and inhibited completely in quantities of 0.25 mg.

Thirdly, a sample of the unsaturated fatty acid fraction was dissolved in ether and shaken with norit to remove the pigment. Most of the pigment was removed. The light yellow oil obtained by evaporation of the ether caused complete inhibition in quantities of 0.25 mg. Therefore one may say that although the pigment follows the activity throughout most of the fractionation it is in itself not the inhibiting agent. However, the last traces of pigment were not removed. Even on distillation the oil that came over had a light yellow color.

Since the active fraction had an iodine number of 90, a sample of the oil was hydrogenated using a platinum black catalyst.² Hydrogenation completely destroyed the inhibiting action of the unsaturated fatty acid fraction. No inhibition was obtained with 5.0 mg., equivalent to 119 mg. of pancreas.

DISCUSSION

The results of these experiments show that the chicken sarcoma-inhibiting factor in pig pancreas is definitely associated with the un-

² The author wishes to thank Dr. E. C. Kleiderer of the Lilly Research Laboratories for carrying out the hydrogenation.

saturated fatty acid fraction. The acid number, the iodine number, and the physical properties are similar to those of oleic acid. This does not necessarily mean that the inhibiting agent is oleic acid. Further purification will be necessary to clear up this point. Commercial oleic acid was found also to exert an inhibiting action against the chicken sarcoma agent in quantities comparable to the fractions isolated from the pancreas. Hydrogenation of the unsaturated fatty acid fraction destroyed its inhibiting properties. These results are in agreement with those of Pirie, who reported that oleic acid inactivated the Fujinami tumor filtrate but that stearic acid did not. However, our findings differ from those of Pirie in that the unsaturated fatty acid fraction was found to exert a markedly stronger inhibiting effect than did the phospholipoid fraction.

The high concentration of lipase in pancreatic tissue may explain the strong inhibiting action of pig pancreas. Without doubt the lipase is responsible for the large quantities of free fatty acid which may be liberated by autolysis after the death of the animal. However, it is interesting to note that sarcoma of the pancreas is rarely found at human postmortem examinations (12).

Along with the work of Begg and Aitken and of Pirie, the data presented in this paper definitely show that the unsaturated fatty acids are able to act directly on the tumor-inducing agent of chicken sarcoma as well as to increase the resistance to transplantable mouse tumors, as shown by Nakahara. More work will have to be done before any conclusions can be drawn as to the mechanism of the inhibiting action of the unsaturated fatty acid fractions.

SUMMARY

The inhibiting action of pancreatic tissue was found to be associated with the unsaturated fatty acid fraction. As small an amount of fatty acid as 0.1 mg. inhibited the chicken sarcoma agent contained in 0.2 cc. of a 1:60 aqueous extract of Chicken Tumor I. The unsaturated fatty acid had an acid number and an iodine number similar to those for oleic acid. Commercial oleic acid also was found to inhibit the growth of the chicken sarcoma in comparable quantities.

The author wishes to thank Dr. L. G. Zervas and Dr. G. H. A. Clowes for their advice and encouragement.

GROWTH-INHIBITING FRACTION OF PANCREAS

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TISSUE CULTURE STUDIES ON BACTERIAL HYPERSENSITIVITY

I. TUBERCULIN SENSITIVE TISSUES

BY JOHANNES K. MOEN, M.D., AND HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATES 22 AND 23

(Received for publication, May 13, 1936)

Tissue culture methods have been but little used in the elucidation of the various problems encountered in bacterial hypersensitivity. The few studies reported have dealt with tissues from tuberculin sensitive animals. Rich and Lewis (1) showed that tuberculin in proper concentration had a selective toxic effect on cells from tuberculous animals. They believed that the tuberculin sensitivity was inherent in the cells, and that the cytotoxic effect was the result of an antigen-antibody reaction. Aronson (2) confirmed this work and also demonstrated the cytotoxic specificity of various tuberculins on sensitive cells as compared with an indifferent effect of extracts made from other acid-fast organisms. In another series of experiments he (3) compared the reaction of tissues from animals sensitized to proteins with that of tissues sensitive to tuberculin when the respective antigens were added to the culture media. Horse serum added to cultures of tissues from horse serum sensitized animals produced no demonstrable cytotoxic effect; this was in marked contrast to the cytotoxic action of tuberculin on tissues from tuberculous animals; he thus showed that there is a fundamental difference between the two types of hypersensitive tissues. We (4) have also noted that cells from animals sensitized with horse serum, egg albumin or beef lens were not specifically inhibited when the respective antigens were added to tissue cultures. As a preliminary to an analysis of other types of bacterial hypersensitive states it was thought advisable to make a detailed study of tuberculin allergy since this is a prototype. This communication deals, therefore, with a partial repetition of previous work with amplifications and extensions.

EXPERIMENTAL

Animals.—The animals were young rabbits weighing from 1,000 to 1,300 gm., and male albino guinea pigs weighing from 300 to 400 gm.

*Tubercle Bacilli.*¹—Three strains of tubercle bacilli were used: B 1, a bovine strain virulent for rabbits; H 37, a human strain, virulent for guinea pigs; and R 1, a human strain of very low virulence even for guinea pigs. The bacilli were grown on plates of Corper's egg medium, harvested, weighed and suspended in normal saline by grinding in a mortar so that 0.2 cc. of the suspension contained the desired amount of organisms for each animal inoculation. Rabbits were inoculated with 0.1 mg. of strain B 1 intravenously, and guinea pigs with 0.1 mg. of strain H 37 or 5.0 mg. of strain R 1, subcutaneously or intratesticularly.

Tissue Culture Media.—Carrel micro flasks and homologous media were used throughout; i.e. normal rabbit plasma with 25 per cent rabbit embryo extract, or 10 per cent rabbit splenic extract for growth of rabbit tissue; and normal guinea pig plasma with 10 per cent guinea pig splenic extract were employed for guinea pig tissues. 4 cc. of blood obtained by cardiac puncture was placed in each chilled tube containing 0.5 cc. of heparin solution (concentration for rabbit blood was 1-1,000 and for guinea pig blood was 1-700 in Ringer's solution²). The tubes were kept cold, centrifuged at high speed, after which the plasma was removed and pooled. Tissue extracts were prepared by finely mincing embryonic or splenic tissue and adding Tyrode's solution to make the required strength. The suspension was thoroughly mixed, allowed to stand for $\frac{1}{4}$ hour, then centrifuged at high speed for a similar period. The clear, slightly opalescent supernatant fluid was used as tissue extract. 1 cc. of normal plasma and 0.5 cc. of tissue extract were used in each flask and mixed just before transferring the explants.

Tuberculin Concentration.—Tuberculin³ suitably diluted in Tyrode's solution was mixed with plasma so that it comprised one-tenth of the total volume of the media in the flask. The final dilution of tuberculin used in the media was from 1-200 to 1-300, as this concentration had but slight inhibitory effect on normal cells.

Explants.—Splenic and testicular explants from both rabbits and guinea pigs

¹ The authors wish to acknowledge the receipt of various strains of tubercle bacilli from the following: Dr. Florence R. Sabin for the H 37 and B 1 strains; Dr. S. A. Petroff, of Trudeau Sanatorium, and Miss Lucy Mishulow, of the City of New York Bureau of Laboratories, for R 1 strains.

² A more highly purified heparin has been obtained from the Connaught Laboratories, Toronto, Canada, and this has been used in later experiments. The most suitable concentration for guinea pig blood was found to be about 0.5 cc. of a 1 to 8,000 solution for 4 cc. of blood.

³ Tuberculin and glycerin broth controls were kindly supplied by Dr. John Reichel, Director of Mulford Laboratories, Sharp and Dohme. The same lot of tuberculin was used throughout these experiments.

were used. Splenic explants, especially from the guinea pig, contained an abundance of wandering cells and fibroblastic elements; they therefore were most satisfactory for our studies, and were most often employed. The animals were killed by a sharp blow over the head; the tissues were removed aseptically; the central portions were cut into explants approximately 1 mm. square and were washed in Tyrode's solution. Four explants were transferred to each flask as soon as the media were thoroughly mixed. Twelve explants were used for each experimental condition. Incubation was carried out at 37.5°C.

Experimental Observations.—Qualitative and quantitative estimations of the effect of tuberculin on sensitive and normal cells were made daily. Microscopic examinations, revealing changes in the size, shape, color and amount of granulation of the cells, indicated the different degrees of toxicity of the tuberculin. Quantitative estimations of the relative increase in areas of wandering cell migration and of fibroblastic growth were determined by ocular micrometric (5) and by projectoscopic methods (6) respectively. The areas of cellular migration were roughly circular; and since the area of a circle is directly proportional to the square of its radius, the square of the average radius of the twelve explants and growths in each experimental set up were compared. Fibroblastic growths were more irregular in outline so that their areas were determined by projectoscopic methods.

Definition of Quantitative Terms Used.—

$$\text{Rate of migration or growth} = \frac{\text{Area of growth} - \text{area of explant}}{\text{Area of explant}} \text{ per unit of time}$$

$$\text{Cytotoxic index} = \frac{\text{Rate of growth in media containing tuberculin}}{\text{Rate of growth in media not containing tuberculin}}$$

If the cytotoxic index is approximately 1, the tuberculin in that concentration has an indifferent effect; if distinctly less than 1, it is toxic.

$$\text{Comparative cytotoxic index} = \frac{\text{Cytotoxic index of tuberculin on test explants}}{\text{Cytotoxic index of tuberculin on normal explants}}$$

If the comparative cytotoxic index is about 1, the test tissue is not sensitive to tuberculin; if definitely less than 1, the test tissue is sensitive or specifically inhibited; in other words, tuberculin has a specific cytotoxic effect on the sensitized tissue.

The initial growth energy refers to the growth rate of tissue in normal media.

$$\text{Comparative initial growth index} = \frac{\text{Rate of growth of test explants in normal media}}{\text{Rate of growth of normal explants in normal media}}$$

RESULTS

Course of the Experimental Tuberculosis.—The bovine strain, B 1 of tubercle bacilli, induced in rabbits a slowly progressive disease involving many viscera, with cachexia and a lethal outcome.

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The H 37 virulent human strain injected subcutaneously into guinea pigs induced a local caseous lesion which usually drained and healed. Progressive involvement of the regional lymph nodes was followed by other visceral disease, and the infection usually terminated fatally after a period of weeks or months. Moderate to marked splenomegaly usually occurred.

The R 1 human strain of low virulence, usually injected intratesticularly, caused a local inflammatory reaction with only moderate general reaction. When this strain was injected subcutaneously, regional lymphadenopathy as well as a local abscess developed. During the acute stage multiple white punctate hepatic lesions and slight to moderate splenomegaly occurred. After the acute local inflammation subsided, the splenomegaly decreased; the animals gained weight and appeared generally healthy. A fatal outcome seldom occurred. The local lesion in the testicle persisted for months as a caseous mass in which acid-fast bacilli in large numbers could be demonstrated.

Tuberculin Reactions in Guinea Pigs.—Positive reactions of the delayed inflammatory type were elicited when 1.0 mg. of human old tuberculin was injected intracutaneously. A maximal reaction was usually reached at 24 hours. Similar skin responses to tuberculin were elicited in animals infected with both strains R 1 and H 37. Seriously ill or moribund pigs usually gave hypoergic reactions.

Histologic Picture of Organs from Which Explants Were Obtained.—Sections of spleens from animals infected with the virulent H 37 strain showed extensive epithelioid cell hyperplasia, especially of the Malpighian bodies, with numerous giant cells. Typical tubercles with areas of central necrosis were frequently found.

Splenic sections from animals infected with strain R 1 showed marked epithelioid cell hyperplasia with numerous giant cells during the early acute toxic phase in which splenomegaly was present. Areas of central necrosis were not noted. The histologic picture during the healing stage, in which the spleen returned to normal size, showed regression of the lesions to a nearly normal picture.

Tissue Culture Observations

Cellular Migration and Fibroblastic Growths from Explants in Normal Media.—Migration of small wandering cells, mostly polymorphonuclear, appeared soon

after explantation of splenic tissue. These cells continued to wander out into the medium away from the explant for about 24 hours, when they began to degenerate and disintegrate. By the 2nd day numerous large wandering cells of the macrophage type were in evidence. With each succeeding day the cells became larger, the protoplasmic processes more complex and the extent of migration greater. The advancing line of cells was usually sharp and the outline roughly circular. After 4 days the cellular migration from explants in normal plasma had usually extended so far that cells from different explants in the same flask intermingled; this rendered further quantitative measurements impossible. Fibroblasts from splenic explants appeared 2 or 3 days after explantation and grew out as solid sheets of cells. Testicular explants produced almost entirely fibroblastic forms with but few scattered wandering cells.

Qualitative Effect of Old Tuberculin on Cells from Normal and Tuberculous Animals.—Preliminary experiments showed that tuberculin in a concentration of 1-300 had but slight effect on cells from normal animals; this visible effect was chiefly a slight increase in the amount of granulation, attenuation of protoplasmic processes, or a slight decrease in cell size. Most of the normal cells in the presence of this concentration of tuberculin appeared healthy and active at the termination of experimental observations.

Cells from tuberculous animals, on the other hand, were severely injured by the tuberoulin.

The more highly sensitive cells migrated only short distances before they died, degenerated and disintegrated. Cells with lesser degrees of sensitivity survived longer and migrated further. Various grades of coarse granulation and vacuolization of the cytoplasm developed; protoplasmic processes shortened; the cells became smaller and rounder; and cellular disorganization was followed by disintegration. The cytotoxic effect of tuberculin on fibroblasts from tuberculous animals was roughly parallel, but as a rule these cells were somewhat more resistant. Figs. 1 to 8 show the comparative effect of tuberculin on sensitive and normal macrophages and fibroblasts. Tuberculin had a similar cytotoxic effect on fibroblastic growth from testicular explants.

Quantitative Determinations of Cellular Migration and Fibroblastic Growth.—The extent of splenic cellular migration was measured daily by means of ocular micrometric methods, and the amount of testicular fibroblastic growth by projectoscopic and planimetric determinations. The measurements of cellular migration from the twelve explants within each experimental condition usually varied but little, so that

Comparative Cytotoxic Index

Duration of Infection in Animals at time of Explantation

Days 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510

● - Index for guinea pig splenic macrophages 4 days after explantation
 ✕ - " " " rabbit testicular fibroblasts 10 " " "
 ○ - " " " guinea pig splenic fibroblasts 10 " " "
 □ - " " " rabbit testicular fibroblasts 10 " " "

Comparative cytotoxic indices of tuberculin for macrophage and fibroblast cultures from tuberculous guinea pigs and rabbits infected with a strain of tubercle bacilli

CHART 1. Comparative cytotoxic indices of tuberculin for macrophage migration or fibroblastic growth for explants from tuberculous guinea pigs and rabbits. The small figure near each index indicates the infecting strain of tubercle bacillus.

CHART 1. Comparative cytotoxicity of tuberculous and fibroblastic growth for explants from various strains of *M. tuberculosis*. The small figure near each index indicates the infecting strain.

were determined 4 days after explantation in the case of splenic wandering cells and 10 days after explantation in the case of testicular fibroblasts. These indices varied from 0.09 to 0.78. Tissues from tuberculous animals were found sensitive as early as 14 days after inoculation. Splenic and testicular fibroblasts were about equally sensitive. Cells from animals infected either with the highly virulent H 37 or with the lowly virulent R 1 strains showed similar degrees of sensitivity to the tuberculin. This marked sensitivity persisted in

cells from R 1 infected guinea pigs at least as long as 4 months after infection, at which time the spleens were essentially normal macroscopically and microscopically; and the only demonstrable foci of infection were small caseous abscesses at the original sites of infection.

Correlation between the Microscopic Appearances and Quantitative Migration and Growth of Cells.—As a rule there was a close correlation between the microscopic appearances of cells and the comparative indices of tuberculin cytotoxicity.

Markedly sensitive cells migrated only slightly in media containing tuberculin and were soon killed; hence comparative indices were correspondingly low. Lesser degrees of sensitivity were manifested by greater activity of the cells and higher indices. A prime requisite for assuring validity of quantitative measurements is the production of firm fibrin clots in which the explants were placed. Firmer clots were obtained when the plasma was but slightly diluted. In instances where excessive fluid collects over the explant some of the surface cells float beyond the margin of cellular migration. With a little experience these cells can be easily recognized and thereby false figures for cellular migration avoided.

Specificity of Tuberculin Toxicity on Sensitive Cells.—In order to test the specific toxicity of old tuberculin on sensitive tissues, other cytotoxic materials such as concentrated glycerin broth, autolysates of various streptococci, streptococcal extracts and proteins were used. Suitable dilution of these substances having but slight effect on normal cells, had little, if any, greater effect on tuberculin sensitive cells, thereby indicating the specific toxicity of tuberculin.

Duration in Vitro of Cellular Sensitivity to Tuberculin.—In view of the persistence of cellular sensitivity to tuberculin months after the acute local inflammatory reaction had subsided in animals infected with strain R 1, it seemed advisable to determine the duration of this sensitivity *in vitro* when cells from tuberculous animals were grown in media containing normal plasma and extract.

Several experiments were undertaken with splenic explants from guinea pigs, 1, 3 and 4 months respectively after intratesticular infection with strain R 1. The two animals infected 3 and 4 months previously were in good general condition, both reacted positively to tuberculin injected intracutaneously, and at autopsy both showed only small caseous tuberculous abscesses in the inoculated testicle. No other macroscopic evidence of tuberculosis was discernible; and, except for slight enlargement of the spleen in one animal, no other abnormality was noted. Smears from the testicular foci showed numerous acid-fast bacilli. Culture of

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finely minced splenic tissue on Corper's egg medium failed to grow tubercle bacilli. Sections of spleen failed to show acid-fast bacilli.

In Experiment 212 the comparative cytotoxic index was 0.29, 4 days after explantation, as shown in Table I, indicating that the cells from the tuberculous animal were markedly sensitive to tuberculin. Fibroblastic growths from the tuberculin sensitive and normal explants, grown in normal plasma without tuberculin, were allowed to proliferate for 10 days, at which time large sheets of cells had formed. Transplants were made from these growths in the usual manner, by selecting actively growing cells toward the periphery and discarding the central portions. These transplants were then placed in new media so that half of each of the sensitive and normal transplants were placed in media containing the same

TABLE I
Persistence in Vitro of Cellular Sensitivity to Tuberculin

Experiment No.	Duration of tuberculous infection (strain R1)	Comparative cytotoxic indices of tuberculin after explantation		Comparative cytotoxic indices after first transplantation of fibroblasts		Comparative cytotoxic indices after second transplantation of fibroblasts	
		Day	Index	Day	Index	Day	Index
212	mos. 4	4th	0.29	5th (15) 7th (17) 9th (19)	0.25 0.28 0.29		
218	3	4th	0.42	4th (15) 7th (18) 9th (19)	0.30 0.28 0.30	4th (25) 7th (28)	0.56 0.41
242	1	4th	0.37	4th (12) 6th (14)	0.32 0.41	4th (20) 6th (22)	0.51 0.72

The numbers in parentheses after the days of transplantation indicate the number of days since the original explantation.

concentration of tuberculin as in the original set up, and the other half of similar transplants were placed in control media without tuberculin. Comparative cytotoxic indices of fibroblastic growth determined on the 5th, 7th and 9th day after transplantation, or, in other words, on the 15th, 17th and 19th day after the original explantation, were 0.25, 0.28 and 0.29 respectively; this showed that tuberculin sensitive cells maintained their sensitivity after proliferation in normal media in tissue culture. The few scattered macrophages carried over with the fibroblastic growths also exhibited sensitivity to tuberculin.

In a similar experiment (218, Table I) explants from another animal, infected with strain R 1 for 3 months, were specifically inhibited and had a comparative index of 0.42. Transplantation of fibroblastic growths was made as before with

resulting comparative indices of 0.30, 0.28 and 0.30 on the 4th, 7th and 9th day respectively. Secondary transplants had comparative indices of 0.56 and 0.41,

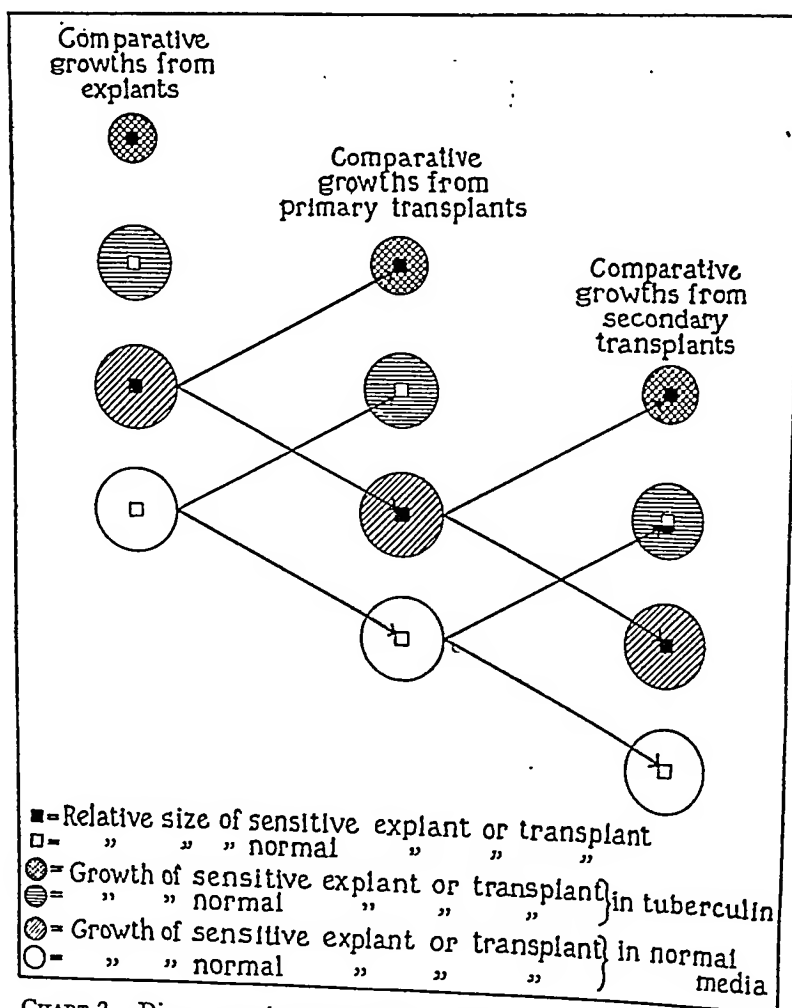


CHART 2. Diagrammatic representation of persistence *in vitro* of cellular sensitivity to tuberculin. The arrows indicate the division of fibroblastic growths into transplants.

on the 4th and 7th day after transplantation, or the 25th and 28th day after explantation, respectively; this indicated that tuberculin sensitive cells still maintained their sensitivity after two transplantations during which time many genera-

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tions of cells must have resulted by proliferation in artificial culture. The course of events in Experiment 218 is graphically represented in Chart 2.

In Experiment 242 (Table I) the animal had been infected with strain R 1 for 1 month. Culture of the minced spleen on Corper's media failed to show growth of tubercle bacilli. A heavy suspension of the minced spleen injected subcutaneously into a normal guinea pig produced no local abscess or adenitis, and autopsy 1 month later showed no macroscopic evidences of tuberculous infection. The comparative cytotoxic index 4 days after explantation was 0.37. On the 4th and 6th day after primary transplantation the comparative indices were 0.32 and 0.41 respectively. On the 4th and 6th day after secondary transplantation, that is, on the 20th and 22nd day after original explantation, the indices were 0.51 and 0.72 respectively.

Experiments 218 and 242 show that there is a very gradual loss of cellular sensitivity to tuberculin on prolonged growth in normal media. In Experiment 242 several of the explant fibroblastic growths in normal media were excised, sectioned and stained for acid-fast bacilli, but careful search failed to reveal their presence. It thus appears in the above experiments that tubercle bacilli were absent or if present were undetectable in the explanted spleens from strain R 1 infected animals, since it has been impossible to demonstrate acid-fast bacilli by culture, by animal inoculation or by stained sections of fibroblastic growths from the explant. It therefore seems improbable that growth of tubercle bacilli in the explanted tissue is the explanation for the persistence of cellular sensitivity to tuberculin *in vitro*.

Comparison of Initial Growth Energy of Tuberculin Sensitive and Normal Explants.—In view of the varying pathological picture of sections of spleens from which explants were taken it seemed probable that there would be a difference in the initial growth energy of the tuberculin sensitive cells, depending on the stage of infection and type of infecting tubercle bacillus. In Chart 3, which shows the comparative initial growth indices of thirteen experiments using guinea pig splenic explants, it is seen that cells from strain H 37 infected animals were less active, with indices varying from 0.28 to 0.53. The activity of tuberculin sensitive cells from animals infected with strain R 1 was moderately retarded early in the course of the infection (indices 0.49 to 0.72) during which time a splenitis was demonstrable. 3 and 4 months after infection when the spleens were practically normal macroscopically and histologically, the growth indices were nearly normal and varied from 0.83 to 1.18. There was thus a close cor-

relation between the pathologic picture of the spleens and the growth capacity of splenic explants in normal media. Explants from spleens exhibiting marked pathological features produced much less vigorous growths than did explants from spleens nearly normal histologically.

Correlation between Initial Growth Energy and Tuberculin Sensitivity of Splenic Explants.—By comparing Charts 1 and 3 it is seen that there is no distinct correlation in this respect. Splenic explants from animals late in the course of the infection with strain R 1, at which time the spleens were nearly normal histologically, were almost as

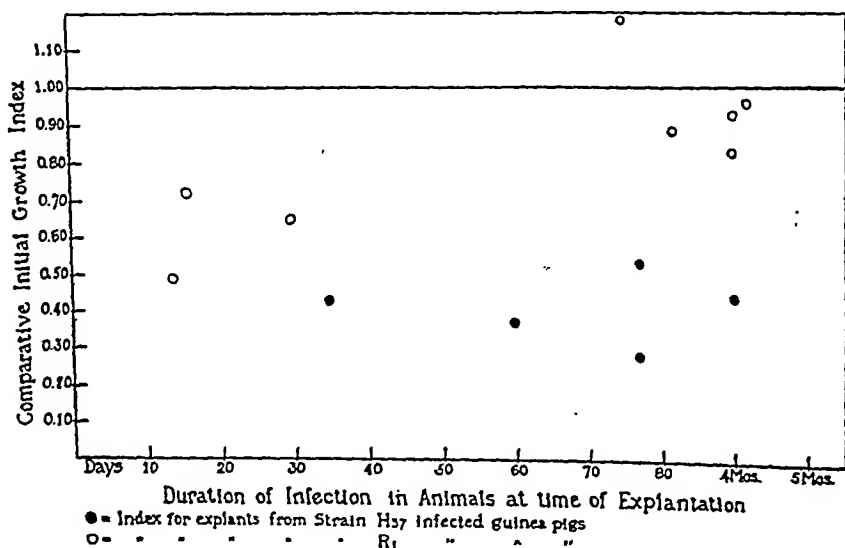


CHART 3. Comparative initial growth (macrophage migration) indices for splenic explants from tuberculous guinea pigs 4 days after explantation.

sensitive to tuberculin as were explants from spleens, pathological in the gross, of guinea pigs infected with strain H 37.

DISCUSSION

These experiments clearly show that human and bovine old tuberculin have a marked specific toxic effect on explanted cells from animals infected with various strains of tubercle bacilli, and corroborate the work of previous investigators (1, 2). There appears to be no definite correlation between the virulence of the infecting tuber-

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cle bacillus or the extent of tuberculous lesions as regards the resulting sensitivity of cells to tuberculin. Cells from animals infected with lowly virulent strain R 1 are nearly as much inhibited by tuberculin as are cells from animals infected with a virulent strain, H 37, in which extensive progressive visceral lesions occur.

The specificity of the cytotoxic effect of tuberculin on sensitive cells has been established by showing that certain other toxic bacterial products do not exhibit this selective inhibition on cells from tuberculous animals. Aronson (2) has also shown that there is a certain degree of specificity of extracts prepared from various acid-fast organisms in regard to their cytotoxic action.

The inherent sensitivity of tuberculin sensitive cells has persisted for at least two transplantations *in vitro*, during which time the original cells proliferated to form many new generations. The original explants from animals infected with strain R 1 were apparently free from living tubercle bacilli since cultures and stained sections of and animal inoculations with, similar portions of the spleens failed to reveal the presence of these microorganisms. These cells, although grown *in vitro* in a medium free from the products of tuberculous infection, other than those present in the explants themselves, continued to possess this tuberculin sensitive characteristic through repeated generations. There was, however, a gradual decrease in the degree of sensitivity on prolonged culture.

If the sensitivity is inherent in the cell, as available evidence indicates, this can be more clearly demonstrated by using smaller aggregates or single cell cultures. Although explants measuring only 1 mm. in diameter are comparatively small, still this mass of tissue is composed of thousands of cells, and complete removal of body fluids by washing in Tyrode's solution seems improbable, except possibly by perfusion. These body fluids carried over with the explants may diffuse into the media as the explants grow, and may possibly play a rôle in sensitizing the newly grown cells. Experiments are in progress to analyze further these problems and also to attempt sensitization of normal cells to tuberculin *in vitro*.

The nature of the specific cytotoxic effect of tuberculin on cells from tuberculous animals also requires further elucidation. Rich and Lewis (1) suggest that it is an antigen-antibody type of reaction.

On the other hand, there is a possibility that the tuberculin cytotoxicity is the result of an additive effect. In this case one must assume that some toxic material similar to tuberculin bathes the cells in tuberculous animals, and when these tissues are explanted into culture media containing tuberculin, a combined action of the tuberculin and this hypothetical toxic substance results. This explanation seems very improbable, however, as specific tuberculin sensitivity can be demonstrated over a wide range of tuberculin concentrations.

The toxic effect of tuberculous infection on body cells, particularly of the wandering or macrophage type, was manifest *in vitro* by decreased activity. Cells from an animal infected with a lowly virulent strain R 1 of tubercle bacillus showed, during the toxic stage when grown in normal media, a decrease in migratory ability of macrophages and a decrease in quantitative fibroblastic growths. A return to practically normal growth energy was demonstrated during the healing stage, or period of regression of lesions. Marked sensitivity was still in evidence, however, when tuberculin was added to the culture media. Therefore the degree of sensitivity of cells from tuberculous animals to tuberculin *in vitro* does not parallel the acuity of the infectious process but represents a more or less permanent characteristic which has been impressed upon the cell as a result of the infection.

SUMMARY AND CONCLUSIONS

1. A high degree of cellular sensitivity to tuberculin toxicity was demonstrated when explants from tuberculous animals were grown in media containing that substance.

2. Similar degrees of sensitivity were noted in cells derived from animals infected with either virulent or relatively lowly virulent strains of tubercle bacilli.

3. The specificity of the tuberculin cytotoxicity was proven by testing with other bacterial cytotoxic materials.

4. Tuberculin sensitive cells grown *in vitro* in normal media showed, when tested with tuberculin, persistence of this cellular sensitivity through several transplantations during which time many new generations of cells developed.

5. There was a depression of the initial growth energy of explants

from animals during the toxic phase of the disease. During the healing stage the initial growth energy returned to normal although marked sensitivity to tuberculin persisted.

6. The degree of cellular sensitivity to tuberculin *in vitro* did not parallel the acuity of the infectious process but represented a more or less permanent acquired characteristic impressed on the cell as a result of the infection.

The authors wish to acknowledge the invaluable technical assistance of Mrs. Jessie C. Hon throughout these studies.

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EXPLANATION OF PLATES

PLATE 22

FIG. 1. Photomicrograph of splenic wandering cells or macrophages derived from a tuberculous guinea pig and growing in media containing human old tuberculin in a concentration of 1-300. 4 days after explantation. Most of the cells are dead and disintegrating. $\times 270$. The animal had been infected 3 months previously with 5.0 mg. of strain R 1 and, at the time of the tissue culture experiment, appeared to be in excellent condition and showed no macroscopic evidence of tuberculosis except for a small caseous abscess in the inoculated testicle.

FIG. 2. Splenic macrophages from the same tuberculous guinea pig as in Fig. 1, but growing in normal media in the absence of tuberculin. 4 days after explantation. The cells are large with long filamentous pseudopodia indicating active migration. $\times 270$.

FIG. 3. Splenic macrophages from a normal guinea pig and growing in media containing old tuberculin 1-300. 4 days after explantation. The cells are still active and healthy and the only sign of inhibition by the tuberculin is slight attenuation of protoplasmic processes. Compare with Fig. 1. $\times 270$.

FIG. 4. Splenic macrophages from a normal guinea pig and growing in normal media showing normal active cells. $\times 270$.

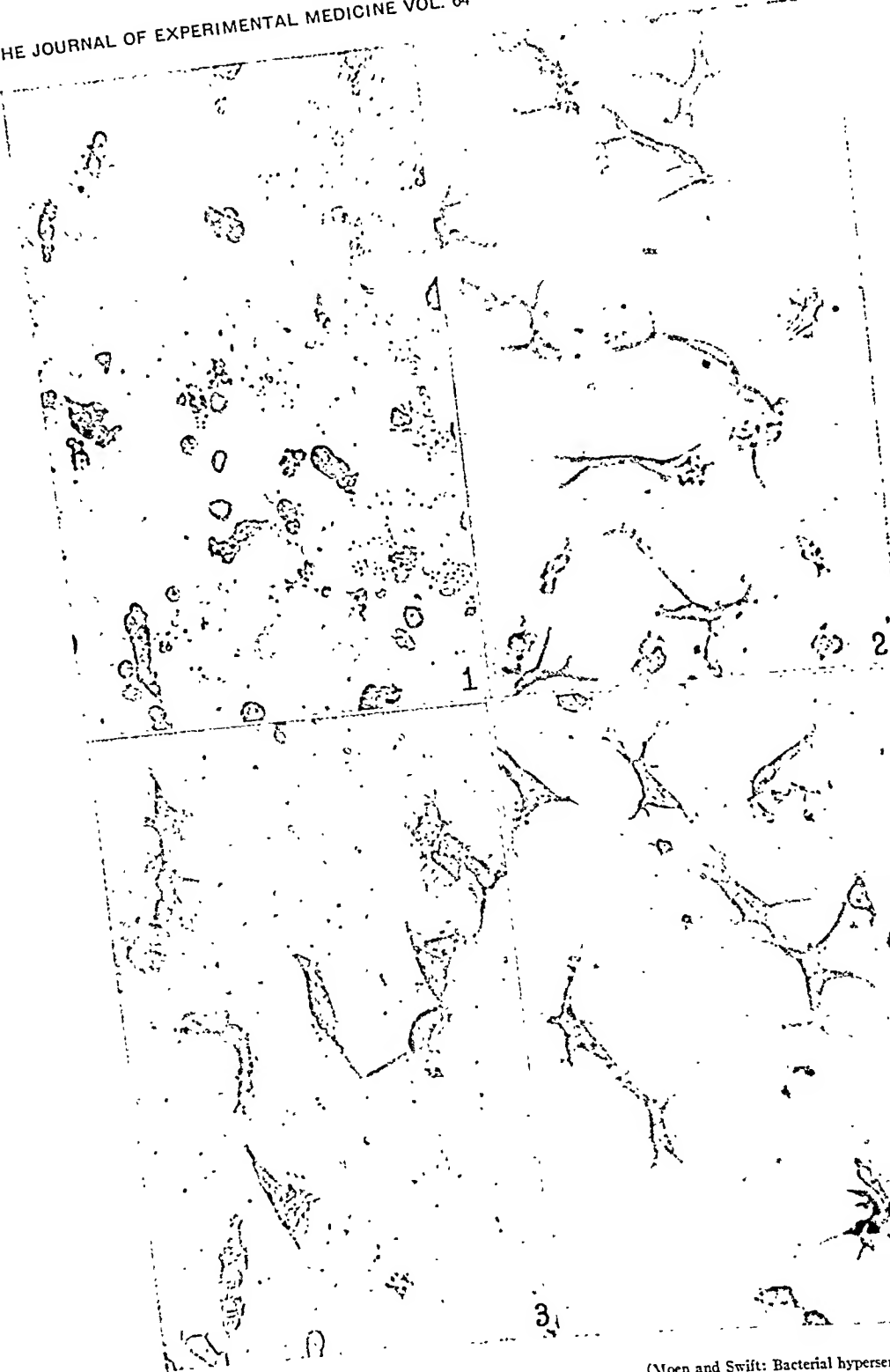
PLATE 23

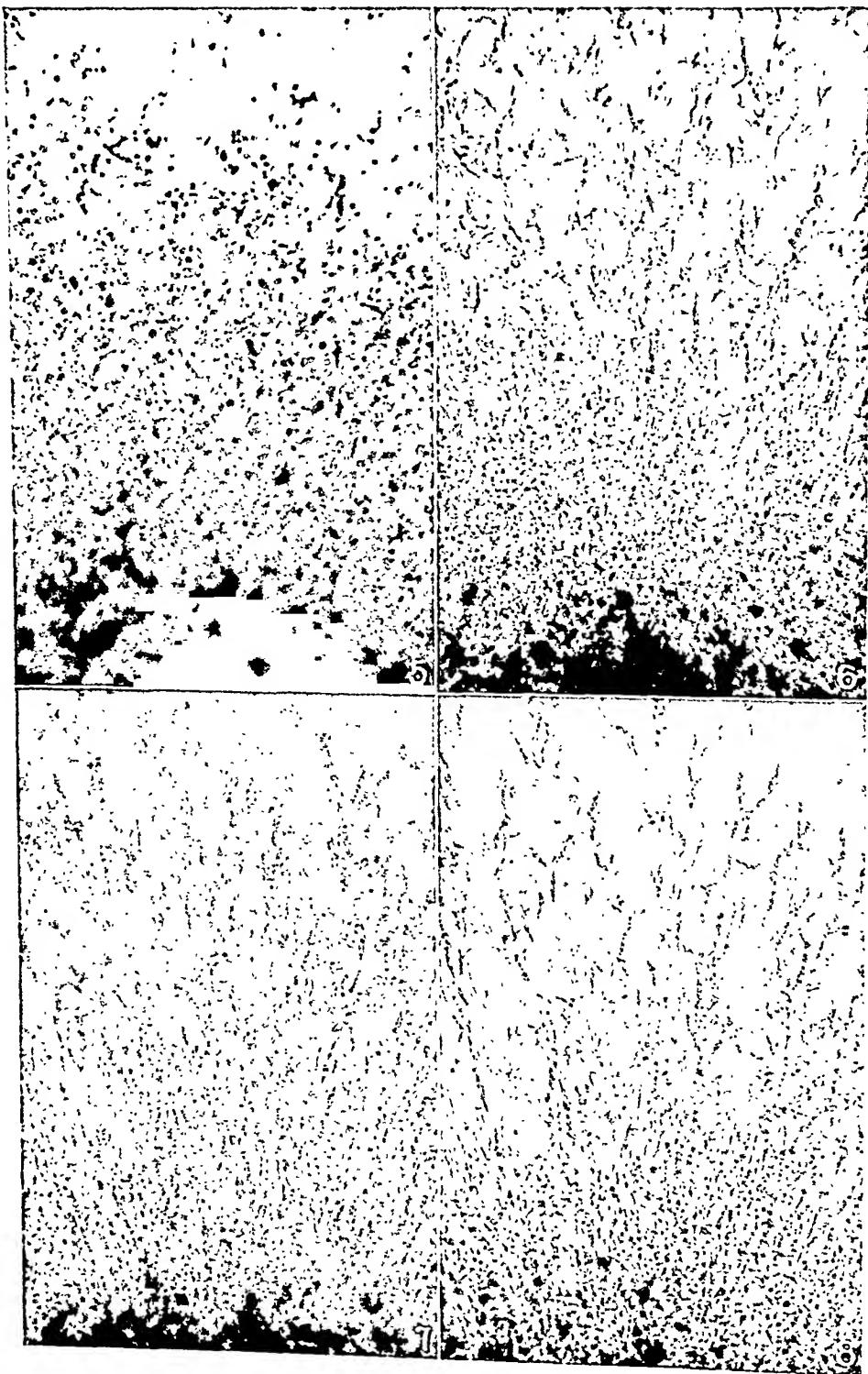
FIG. 5. Lower magnification of same sensitive explant as in Fig. 1, showing marked suppression of fibroblastic growth by old tuberculin 1-300. 4 days after explantation. $\times 75$.

FIG. 6. Lower magnification of sensitive explant in Fig. 2, showing good fibroblastic growth in normal media 4 days after explantation. $\times 75$.

FIG. 7. Lower magnification of normal explant in Fig. 3, showing good fibroblastic growth in media containing old tuberculin 1-300. $\times 75$.

FIG. 8. Lower magnification of normal explant in Fig. 4, showing good fibroblastic growth in normal media 4 days after explantation. $\times 75$.





(Moen and Swift: Bacterial hypersensitivity. I.)

TISSUE CULTURE STUDIES ON BACTERIAL HYPER-SENSITIVITY

II. REACTIONS OF TISSUES FROM GUINEA PIGS INFECTED WITH GROUP C HEMOLYTIC STREPTOCOCCI

By JOHANNES K. MOEN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, May 13, 1936)

Observation of the specific toxic effect of bacterial products on living sensitive cells in tissue culture offers a direct approach to a study of certain phases of bacterial allergic or hypersensitive states. In a preceding study (1) tuberculin was shown to have a specific toxic effect on sensitive cells from animals infected with several strains of tubercle bacilli having various degrees of virulence.

This communication presents the results of an investigation by the tissue culture technique, of hypersensitive states associated with infection by a different species of microorganism, namely the hemolytic streptococcus. This permits of a comparison of cutaneous reactivity, antibody production and the development of cellular susceptibility to the toxic action of certain bacterial products *in vitro*. A comparison with some features of tuberculin allergy can thus be made.

EXPERIMENTAL

Animals.—Albino guinea pigs, mostly males, weighing between 300 to 450 gm. were used throughout, because the strains of hemolytic streptococci employed were natural pathogens for these animals.

Hemolytic Streptococci.—Three strains of hemolytic streptococci, K 104, K 64 and J 20, all isolated from epidemics of spontaneous guinea pig lymphadenitis, and all belonging to group C of Lancefield's serological classification (2) were used. In most experiments strain K 104¹ was employed. The inoculum consisted of 0.1 cc. of an 18 hour broth culture injected subcutaneously in the inguinal region.

¹ Kindly supplied by Dr. Theobald Smith, and originally isolated from spontaneous guinea pig lymphadenitis by Dr. J. G. Hardenbergh, of the Mayo Foundation.

Bacterial Extract.—The bacterial extract for skin testing and for use in tissue culture experiments was derived from the above mentioned microorganisms. After growing in broth culture for 18 hours, the bacteria were thrown down by centrifugation, frozen and dried by a technique previously described by Swift (3) and ground in a ball mill for from 3 to 5 weeks to disrupt completely the organisms. A 0.5 per cent solution of the dried powder was made up in Tyrode's solution, and centrifuged at high speed for 30 to 45 minutes to remove the insoluble portion. The resulting clear, slightly opalescent solution was the bacterial extract used in the experiments.

Tissue Culture Media.—Heparinized guinea pig plasma obtained by cardiac puncture and 10 per cent guinea pig splenic extract were prepared as described in the preceding paper (1). The bacterial extract, in proper dilution, added to the plasma for testing was equal to one-tenth of the total volume of the media. Thus, the final set up consisted of 0.85 cc. of plasma, 0.15 cc. of bacterial extract dilution and 0.5 cc. of 10 per cent guinea pig splenic extract. Carrel micro flasks were used throughout.

Explants.—Splenic explants were used exclusively, since this tissue contained many wandering cells of the macrophage type and also numerous fibroblastic elements. Four explants about 1.0 mm. square were placed in each flask after the culture media had been thoroughly mixed. Twelve explants were used for each experimental condition. Incubation was carried out at 37.5°C.

Experimental Observations.—Qualitative microscopic and quantitative measurements of the wandering cell migration and fibroblastic growths were made daily as described in the preceding paper (1). Qualitative evidences of cellular injury were manifested by changes in size, shape, color, amount of granulation, and by signs of cellular disintegration. The activity of the cells was measured quantitatively as increase in areas of macrophage migration or fibroblastic growth. For a full explanation of the quantitative terms employed such as: rate of growth, cytotoxic index, comparative cytotoxic index and initial growth energy reference should be made to the preceding paper (1).

RESULTS

Course of Experimental Hemolytic Streptococcal Infection in Guinea Pigs.—Obvious local infection almost invariably occurred when 0.1 cc. of an 18 hour broth culture of a group C hemolytic streptococcus was injected subcutaneously. Following an intense local inflammatory reaction, an abscess formed and usually drained spontaneously after 1 or 2 weeks. Within 4 or 5 days local lymphadenitis was demonstrable. The nodes gradually increased in size, measured 1 to 2 cm. in diameter, and became filled with thick purulent material. Distant lymph nodes occasionally were involved. Chronic purulent lymphadenitis usually persisted for weeks or months, and only occasionally did this chronic form resolve by rupture with spontaneous healing. Other lesions occasionally encountered were retroperitoneal abscesses, hepatic abscesses, mediastinal lymphadenitis, areas of pul-

monary consolidation and purulent pericarditis, from all of which hemolytic streptococci were demonstrable on blood agar plates.

Early in the course of the infection, the animals developed fever and lost weight; then they gained weight, appeared active and healthy except for the enlarged lymph nodes. Animals seldom died during the acute stage, and tolerated the chronic infection quite well.

Skin Reactivity to Bacterial Extract.—A delayed inflammatory reaction similar to a positive tuberculin test developed when 0.1 cc. of the bacterial extract, containing the soluble products of 0.5 mg. of the ground bacteria, was injected intradermally into infected guinea pigs. The reaction, which usually reached its height at 24 hours, was characterized by redness, edema, induration and in some instances by areas of central necrosis. Involution required several days; and when central necrosis eventuated a persistent scar developed. Normal animals failed to react to this dose of bacterial extract. Repeated skin testing in the same noninfected animal would, however, result in a positive skin reaction to subsequent testing.

Cutaneous hyperreactivity was demonstrable as early as 5 days after infection. In general, the most intense reactions, consisting of large, very red, edematous and indurated lesions, occurred in the period 2 to 3 weeks after infection. This was followed by a decrease in cutaneous reactivity, although large foci of infection persisted. Cutaneous hypersensitivity, of diminishing intensity, persisted in 24 guinea pigs in which spontaneous healing of the focal lesions had occurred, and which showed no macroscopic lesions at autopsy.

Specificity of Skin Reactivity to Group C Hemolytic Streptococcal Extract.—Several infected animals were also tested with bacterial extracts prepared from a group B hemolytic streptococcus and from a green streptococcus obtained from a guinea pig. Slight reactivity, less than that to the homologous extract, developed, which indicated that some of the reactivity was probably due to a chemical fraction common to the various streptococci used.

Gross and Microscopic Appearances of Spleens Used for Explants.—The spleens from the infected guinea pigs were usually only slightly swollen and softer than normal. Microscopically there was slight to moderate hyperplasia of the reticulum and increase in polymorphonuclear leucocytes in the sinusoids, especially early in the course of the infection. Occasionally there was a slight hyperplasia of cells in the Malpighian bodies.

Tissue Culture Observations

Growth from Explants in Normal Media Not Containing Hemolytic Streptococcal Extract.—Cellular migration from splenic explants derived from both normal and infected guinea pigs when grown in normal media was essentially the same qualitatively.

During the first 24 hours small wandering cells or microphages were most prominent, but by the 2nd day many of them were undergoing degeneration and

disintegration. The large wandering cells or macrophages continued to migrate outward, with a roughly circular advancing border. Each day the area of cellular migration increased so that by the 4th day the areas from the four different explants were nearly contiguous. On the 2nd or 3rd day after explantation, spikes of fibroblastic growth extended out from the explants; and growth continued until large circular sheets of fibroblasts were formed. Experimental observations were usually terminated after the 4th day, at which time most of the macrophages were still healthy and active, and vigorous fibroblastic growths were present. It was frequently observed that the wandering cells of the macrophage series from spleens of infected pigs had a more stimulated appearance and migrated farther than did those from normal animals, thus showing a greater initial growth energy of cells from streptococcal infected animals.

Growth from Explants in Media Containing Hemolytic Streptococcal Extract.—Within a few hours after explantation there was a distinct stimulation of small migrating cells from all explants in media containing the streptococcal extract in a concentration of 1-6,000. This was a constant occurrence in all experiments and was seen in explants from both normal and diseased animals. For example, measurements 7 hours after explantation in a typical experiment showed that the areas of cellular migration from explants in media containing the bacterial extract were more than twice as great as from explants in normal media; this demonstrated the early stimulating or chemotactic effect of the bacterial extract.

A difference in the reactivity of macrophages, from normal and infected animals, was discernible 24 to 48 hours after explantation into media containing the streptococcal extract. The normal cells were only slightly inhibited by the concentration of bacterial extract employed. The cells from infected animals, on the other hand, became rounder, darker, more granular and their migration was distinctly inhibited. With each succeeding day the differences in the degree of migration became more marked, due to the specific inhibitory or cytotoxic effect of the bacterial extract on the latter. 4 days after explantation, at which time experimental observations were usually terminated, many of the sensitive cells were dead and disintegrating, while the remaining ones were very dark, granular and inactive. In contrast, most of the normal cells were still healthful in appearance and maintained their active migration. Fibroblastic growths from sensitized explants appeared less inhibited by the bacterial extract than

were the macrophages. The degree of the specific cytotoxicity of bacterial extract varied in different experiments depending to a certain extent on the time relationships after infection. The more highly sensitive cells were rapidly killed; various gradations were seen so that the lesser sensitized cells manifested only slight inhibition in their migratory propensities.

Quantitative Comparisons of Cellular Migration.—A quantitative expression of the comparative inhibitory effect of hemolytic strep-

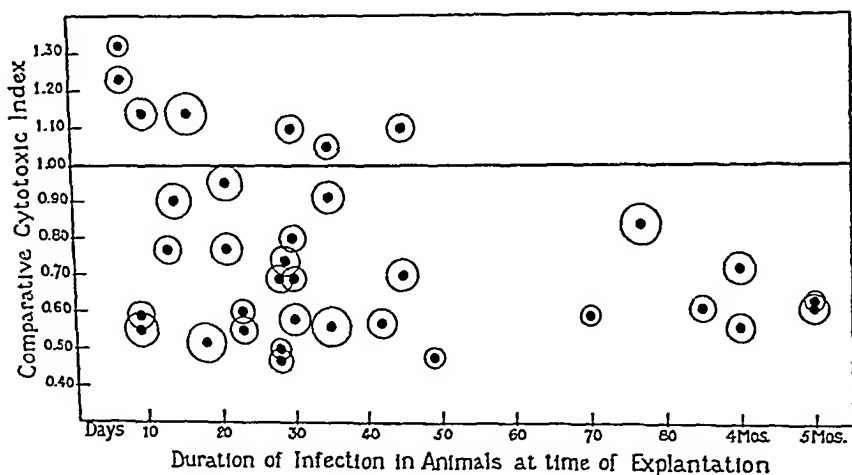


CHART 1. Comparative cytotoxic indices of streptococcal extract for macrophage migration from splenic explants derived from guinea pigs infected with group C hemolytic streptococci. 4 days after explantation. The circle around each index indicates the comparative size of the cutaneous reaction 24 hours after the intradermal injection of streptococcal extract. Skin tests were applied to the animals 1 or 2 days preceding explantation experiments.

tococcal extract on cells from hypersensitive and normal animals was determined in a manner similar to that employed in the previous study on tuberculin sensitive tissues (1). The concentration of bacterial extract which had but slight inhibitory effect on normal cells was determined by preliminary experiment to be about 1-6,000. If the comparative cytotoxic index was distinctly below 1, a specific inhibitory or toxic effect on the sensitized cells was indicated.

Since the results obtained on the 4th day after explantation repre-

sent the maximal demonstrable effect of the bacterial extract on sensitized and normal cells, comparative cytotoxic indices of 35 experiments for that day are shown in Chart 1. Each dot represents the comparative cytotoxic index for a single experiment, in each of which 48 explants were used, and is plotted against the duration in days after the respective animal was infected. 28 of the 35 indices are below 1, and seven are above. Four of this seven above 1 occur between the 7th and 16th day after infection. After the 45th day all indices are definitely below 1. Chart 1 shows that cells from most of the streptococcal infected animals were specifically inhibited by the

TABLE I

Specificity of the Toxic Effects of Old Tuberculin and Streptococcal Extract on Sensitive Cells in Vitro

	Index	
Comparative cytotoxic index of old tuberculin on cells from tuberculous animal	0.39	Indicates specific toxic effect of old tuberculin on tuberculin sensitive cells
Comparative cytotoxic index of streptococcal extract on cells from tuberculous animal	1.02	Indicates indifferent effect of streptococcal extract on tuberculin sensitive cells
Comparative cytotoxic index of streptococcal extract on cells from streptococcal infected animal	0.57	Indicates specific toxic effect of streptococcal extract on streptococcal sensitive cells
Comparative cytotoxic index of old tuberculin on cells from streptococcal infected animal	0.99	Indicates indifferent effect of old tuberculin on streptococcal sensitive cells

homologous bacterial extract in tissue culture. Of five animals tested during the first 10 days after infection only two had indices below 1; this indicates that the time interval after infection is an important factor in the development of sensitive cells.

In general there was a close correlation between the qualitative microscopic appearances and the quantitative estimation of cellular migration as evidences of the cytotoxic effect of bacterial extract.

Specificity of the Cytotoxic Effect of Bacterial Extract on Sensitive Cells.—Bacterial extracts prepared from a group B hemolytic streptococcus and from a strain of *Streptococcus viridans* obtained from a guinea pig had little, if any, greater cytotoxic inhibiting influence on

explants from group C streptococcal infected pigs than on normal explants. Bacterial extracts from closely related strains of streptococci within group C had similar specific cytotoxic effects on explants derived from guinea pigs sensitized by a group C streptococcus. In order to test further whether the toxic effect of bacterial extract on sensitive cells was specific and not simply the result of greater susceptibility to any cytotoxic agent, an experiment was performed involving tests of tuberculin sensitive, streptococcal sensitive, and normal cells with both tuberculin and streptococcal extracts. Results showed that each cytotoxic agent exhibited a specific effect only on the cells sensitized by the corresponding infection. Table I shows the comparative cytotoxic indices of each of the test substances on each of the test tissues; these indices corroborate the qualitative or microscopic changes observed. This experiment clearly demonstrates the specific toxic influence of these bacterial derivatives on the correspondingly sensitive cells and shows that the effect is not due to a heightened vulnerability of these cells to any nonspecific cytotoxin.

Correlation between Cutaneous Reactivity and Cellular Sensitivity to Bacterial Extract in Tissue Culture.—

In order to compare the intensity of two unlike, but possibly related, bacterial hypersensitive reactions, it was necessary to employ two different quantitative measurements. Cutaneous reactivity was represented by the size of skin lesions obtained 24 hours after the intradermal injection of 0.1 cc. of bacterial extract. Following this procedure tissue culture experiments were performed. The degree of cellular sensitivity was indicated by comparative cytotoxic indices determined 4 days after explantation. The cutaneous reactivity is expressed graphically in Chart 1 by determining the average of the two diameters of each skin lesion, by using an arbitrary scale, and by drawing a circle around each comparative index. This offers an opportunity for rough comparison of the relative size of skin lesions, although it is realized that cognizance should also be taken of the amount of edema, induration and central necrosis for complete evaluation of skin reactivity.

Chart 1 indicates that the larger skin reactions developed during the 2nd and 3rd week after infection, and this fact was substantiated by cutaneous reactions of animals not subjected to tissue culture experiments. It is also seen that the size of skin lesions did not parallel the degree of cellular sensitivity to bacterial extract as indicated by the indices. All of the infected animals gave positive skin tests. Later in the course of the infection the skin reactivity decreased but the

cellular sensitivity to the specific toxic action of bacterial extract remained about the same.

Comparative Initial Growth Energy of Explants from Infected and Normal Animals.—It was soon noted that when macrophages from infected animals were grown in normal media, these cells showed greater migratory activity than did normal cells in the same media. Chart 2 shows the comparative initial growth indices on the 4th day after explantation, plotted against the duration of infection in days. An index greater than 1 indicates that the cells from the infected

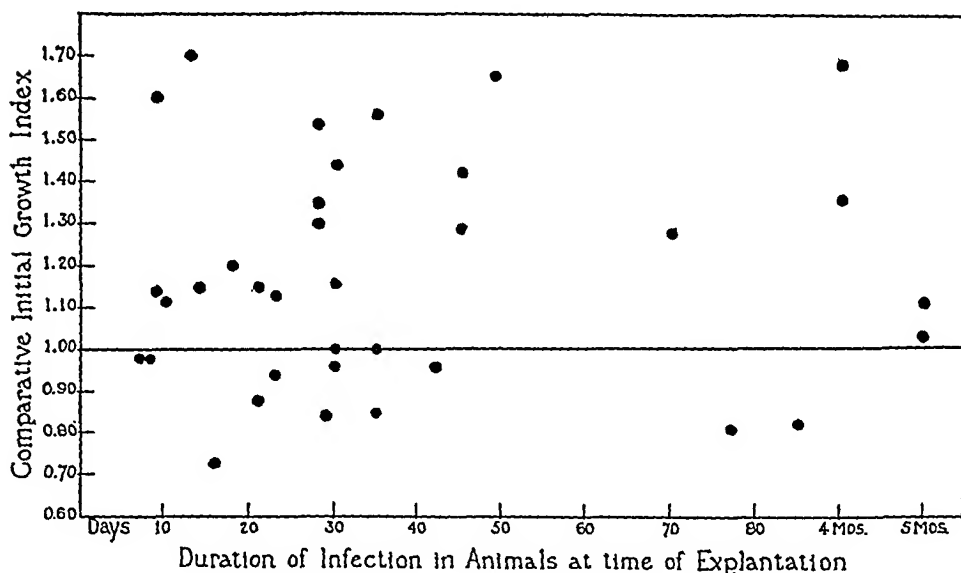


CHART 2. Comparative initial growth (macrophage migration) indices for splenic explants from streptococcal infected guinea pigs. 4 days after explantation.

animal were stimulated; and an index definitely less than 1 that the cells were less active than normal. The comparative indices of 35 experiments varied from 0.73 to 1.70 with an average of 1.19; 24 indices were 1 or greater, and 11 were below 1. Of the 11 below 1, 9 had an index above unity on the 1st or 2nd day after explantation, while only the remaining 2 had indices that persisted below 1 during each of the 4 days following explantation. Thus, stimulation of macrophage activity at least as regards its migratory ability was a usual resultant of infection with the streptococcus under investigation.

Duration of Sensitivity to Specific Toxicity of Bacterial Extract on Transplantation of Sensitive Tissues.—Transplantations of fibroblastic growths were made similar to those in the study of tuberculin allergy (1). There was but little difference between the reaction of fibroblasts transplanted from sensitive explants and from normal explants when tested with bacterial extract. This indicates that sensitivity to this streptococcal extract is not as persistent as sensitivity to tuberculin.

Antibody Studies

Studies on the formation of humoral antibodies with relation to the events occurring during the course of the infection were conducted.

Agglutination Technique.—The group C hemolytic streptococcus (K 104) used in most of the experiments grew diffusely in broth with little tendency to settling. Serum dilutions ranging from 10 to 5,120 were made in normal saline. Living 18 hour broth cultures of streptococci were centrifuged at high speed and re-suspended in saline so that 1 drop of the bacterial suspension in 1 cc. of the serum dilution produced a slightly turbid suspension. Small colony forms of streptococci selected from blood plates were used for seeding broth cultures for use in agglutination. The larger mucoid colonial variants were relatively inagglutinable. The tubes were incubated in a water bath at 37°C. for 2 hours, stored in a refrigerator overnight and read for macroscopic agglutination.

Agglutination Titers.—Sera from 32 animals with active infection had agglutination titers varying from 20 to 1,280 with an average of 430. As shown in Chart 3, all of the lower titers occurred early in the course of the infection, while after the 28th day all titers were above 320. The agglutination titer of 21 animals with apparent recovery from infection varied from 80 to 640 with an average of 210.

Seventeen normal control animals had titers varying from 0 to 80 with an average of 43. One control animal which reacted negatively to an initial skin test, but later gave a positive reaction to a second skin test, had a titer of 160.

Specificity of the Agglutination Reaction.—Comparative agglutination with group A, B and C hemolytic streptococci showed high titers for the serum with its homologous streptococcus and only slight agglutination with streptococci belonging to the other groups.

Precipitating Antibodies.—The 1-200 solution of bacterial extract in Tyrode's used for skin testing and in the tissue culture experiments was used as the pre-

cipitinogen. 0.3 cc. of serum was mixed with 0.3 cc. of dilutions of bacterial extract (1-200, 1-1,000, 1-5,000 and 1-25,000) incubated in the water bath at 37°C. for 2 hours, stored in the refrigerator overnight and read macroscopically.

Precipitin Reactions.—Slight but definite amounts of precipitate developed in 29 of 36 sera (81 per cent) from animals with active infection. The prozone phenomenon was frequently observed. All of the negative precipitin reactions occurred early in the course of the

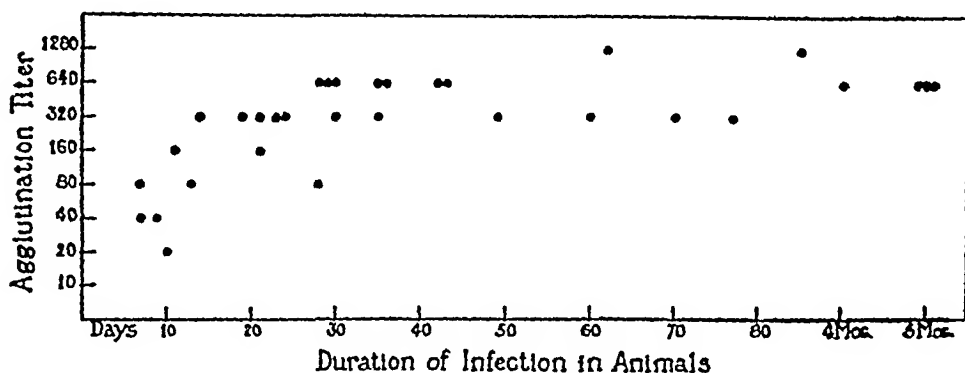


CHART 3. Agglutination titers of sera from hemolytic streptococcal infected guinea pigs.

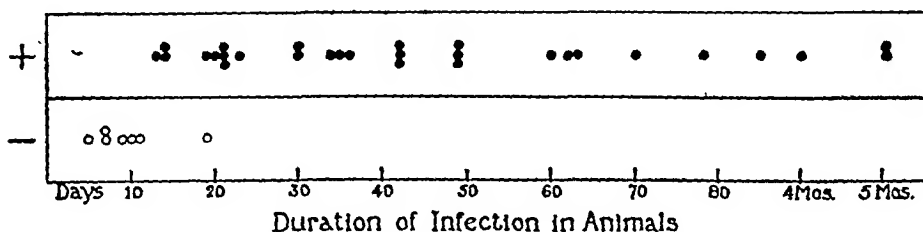


CHART 4. Precipitation reactions of sera from hemolytic streptococcal infected guinea pigs against streptococcal extract.

infection; in fact six of the seven, as shown in Chart 4, fell within the first 11 day period. Five of 22 guinea pigs that apparently recovered from infection, or 23 per cent, gave positive reactions.

Only one of 20 control animals gave a positive precipitin reaction, and this was the same animal that received repeated skin tests and that had a slightly elevated agglutination titer.

Relationship between Time of Infection, Development of Humoral Antibodies and Degree of Skin Reactivity.—The earliest appearance of

precipitins was on the 13th day after infection and about the same time significant increases in the agglutination titer were observed. Since skin hypersensitivity to bacterial extract was demonstrated as early as 5 days after infection, it seemed apparent that there was no close correlation between the appearance of circulating antibodies and the development of skin hyperreactivity. There was also no parallelism between the degree of skin reactivity and the height of agglutination titer when the chronic stage of the infection was established.

DISCUSSION

Sensitization of tissues to bacterial products probably occurs in many infections. The importance of studying bacterial hypersensitive states in experimental animals using bacteria that are natural pathogens is obvious, since this more closely approaches conditions as they occur in nature. The strains of hemolytic streptococci used in the present study were such natural pathogens for guinea pigs.

An analysis of the course of events including tissue culture observations was made following infection of guinea pigs with this micro-organism. The delayed inflammatory type of skin hypersensitivity to the bacterial extract was demonstrable as early as 5 days after infection; on the other hand, circulating precipitins and increase in agglutinins were not detectable until about 2 weeks after infection. The sensitivity of splenic explant cells from infected animals to bacterial extract was made evident *in vitro*, by various degrees of cellular injury. Quantitative measurements of macrophage migration and of fibroblastic proliferation demonstrated the inhibitory effect of bacterial extract on the growth of sensitive cells. The specificity of this toxicity was proven by comparing it with the effect of other bacterial cytotoxic agents.

No correlation was found between the degree of cutaneous reactivity to a given bacterial extract and the degree of sensitivity of splenic explant cells to the same extract *in vitro*. This suggests that skin reactivity is not a reliable index of the degree of sensitivity of internal cells. In these experiments the macrophages were found to be more sensitive to the specific toxicity of a crude bacterial extract than were the fibroblasts; this indicates that different cellular types within the

same animal exhibit different degrees of vulnerability to the same injurious agent. There was also no parallelism between the bacterial allergic skin reactivity and the amount of circulating antibody, a phenomenon which has been noted by others (4-8).

The lack of correlation between the degree of cutaneous reactivity to bacterial extract and the degree of sensitivity of cells to bacterial extract *in vitro* suggests that possibly different chemical fractions of the extract may be involved in the two reactions. This viewpoint is supported by the observation that extracts of streptococci from other immunological groups gave positive cutaneous reactions when tested in animals infected with group C hemolytic streptococci but failed to exert a specific toxic effect when these heterologous group extracts were tested *in vitro* on cells sensitive to group C streptococcal extract. Other experiments (9) show that plasmas from infected animals containing immune bodies neutralize the toxic effect of bacterial extract on sensitive cells *in vitro* but fail to neutralize the substance in bacterial extract that induces cutaneous reactivity in sensitive animals. Recently, Cooke (10) and his coworkers demonstrated the coexistence of immune and skin sensitizing antibodies in the sera of pollen treated hay fever patients. They also noted clinical improvement with production of immune antibodies, although skin sensitivity was not altered, after pollen therapy.

Comparison of tuberculin allergy (1) with group C hemolytic streptococcal allergy in guinea pigs shows many similarities and certain differences. The type of skin reactivity, and of cellular sensitivity to bacterial products *in vitro* are similar. Quantitatively, cellular sensitivity to tuberculin is more intense and persists longer as evidenced by transplantation experiments. The initial growth energy of explants from tuberculous animals is decreased during the acute toxic phase, while, on the other hand, the growth energy of explants from group C streptococcal infected animals is usually increased. This indicates that this particular hemolytic streptococcal infection stimulates cellular activity, at least from splenic explants.

Martin (11) has recently reviewed the literature on investigations by various workers, which show that allergy and immunity can be dissociated and that the former is not necessary for the proper func-

tioning of the latter. The results of these experiments are in accord with the growing opinion that bacterial hypersensitivity or allergy is a harmful state since cells from infected animals display increased vulnerability to the toxic effect of bacterial products. Further studies on bacterial hypersensitivity by the tissue culture technique with particular reference to the mechanism whereby an infected animal may protect its sensitive cells from the toxic effect of bacterial products are in progress.

SUMMARY

1. Guinea pigs infected with naturally pathogenic hemolytic streptococci (group C—Lancefield) develop a low grade chronic type of disease characterized chiefly by purulent lymphadenitis.

2. Cutaneous hyperreactivity to a crude streptococcal extract invariably occurred during the course of this infection.

3. Production of antibodies (precipitins and agglutinins) was studied.

4. The hemolytic streptococcal extract had a specific toxic effect, when tested *in vitro*, on cells from infected animals; this was shown by microscopic evidence of cellular injury, and by quantitative inhibition of cellular migration and growth. The specificity of the reaction was proven by testing with other cytotoxic substances.

5. There was no parallelism between skin hypersensitivity and humoral antibody titer.

6. There was no correlation between the degree of skin reactivity to the bacterial extract and the degree of sensitivity of splenic cells to the toxic action of the same extract *in vitro*.

7. Comparison of cellular sensitivity to tuberculin with cellular sensitivity to streptococcal extract in cultures of guinea pig tissues showed that the former was more intense and was more persistent on prolonged growth *in vitro*.

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THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM IN MICE

IV. THE PROZONE

BY KENNETH GOODNER, PH.D., AND FRANK L. HORSFALL, JR., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, May 28, 1936)

For each lot of antipneumococcus horse serum there is a definite optimal amount which confers on mice the maximal degree of protection against large numbers of pneumococci (1). With amounts of serum greater than this optimum, little or no protection is obtained. This negative effect is described as the "prozone." Certain observations have been reported as to the mechanism underlying this phenomenon (2), but a final analysis was not undertaken because of the curious fact that with antipneumococcus rabbit serum this prozone effect is not obtained.

The present report consists of a comparative study of the protective action of antipneumococcus horse and rabbit serum in mice.

EXPERIMENTAL

Methods.—The details of the general experimental methods have been given in a preceding paper (2), and may be summarized as follows: At determined intervals after infective inoculation, individual mice were sacrificed by deep ether anesthesia and immediately after death the peritoneal cavity of each animal was thoroughly washed out with 1.0 cc. of saline. Leukocyte counts were made on this fluid by the method commonly used for blood. Films of the peritoneal washings were stained by the Gram technic, and examinations made of a large number of contiguous microscopic fields. The numbers of extracellular and intracellular pneumococci were recorded, and also in the same fields the numbers of white cells showing phagocytosis and those which did not contain bacteria were noted. From these data the number of pneumococci per cubic millimeter of peritoneal washings could be calculated with reference to the determined number of white cells.

The antipneumococcus horse and rabbit sera employed in these experiments possessed equivalent antibody content. The "optimal protective quantity" in each case was 0.05 cc. In order to demonstrate the prozone in the case of im-

mune horse serum, 0.4 cc. was used. The infective inoculum in each instance was 0.1 cc. of an 18 hour blood broth culture of a highly virulent Type I Pneumococcus. The total volume of each serum-culture mixture was 1.0 cc. Each

TABLE I

Summary of Observations on the Protective Action of Antipneumococcus Horse and Rabbit Sera

Observation	Time after infective inoculation	0.4 cc. of serum		0.05 cc. of serum	
		Horse	Rabbit	Horse	Rabbit
Unsacrificed control mice					
Total No.		19	11	62	43
No. surviving		1	11	45	39
Survival rates, per cent		5	100	73	91
Mean per cent of the total pneumococci found intracellularly	15 min.	2	2	11	6
	1 hr.	3	7	39	40
	2 hrs.	4	38	36	69
	4 "	24	97	97	100
Extracellular pneumococci per c.mm. of peritoneal washings	15 min.	26,390	19,440	20,800	19,210
	1 hr.	25,740	40,020	4,070	20,600
	2 hrs.	101,980	33,740	20,580	9,720
	4 "	215,460	1,370	690	0
Average No. of white cells per c.mm. of peritoneal washings	15 min.	2,320	2,230	1,970	2,970
	1 hr.	2,890	1,880	1,500	2,770
	2 hrs.	2,280	3,940	2,780	2,430
	4 "	5,340	12,370	8,900	8,160
Mean per cent of white cells active as phagocytes	15 min.	3	6	7	8
	1 hr.	3	19	15	17
	2 hrs.	9	31	5	26
	4 "	34	17	5	7
Mean phagocytic indices (No. of engulfed pneumococci per active phagocytic cell)	15 min.	14	3	43	6
	1 hr.	7	10	14	24
	2 hrs.	22	27	34	36
	4 "	33	16	14	13

figure in the various protocols represents the mean of determinations on four or more mice.

Experiments were arranged to compare the course of the infectious process under four conditions, *viz.*, with optimal and with excess

amounts of horse and rabbit immune sera. The results of these studies are shown in Table I. Figures which are considered more noteworthy for reasons to be cited, are shown in bold face type.

On the basis of our present knowledge a complete analysis of these findings is exceedingly difficult, since many of the factors involved in the infectious process are often confusingly interrelated. It will suffice, however, to point out the essential facts and correlate these with the variables which appear to be related to the nature and amount of the immune serum.

1. It will be noted that the survival rates were high except with the excess or prozoning amount of immune horse serum.

2. The rate at which phagocytosis was accomplished was different for each amount of serum. There is a particularly striking differentiation, for example, at 1 hour. It will be noted that at this time, with both horse and rabbit sera, a large amount delayed the rate of phagocytosis. After this time period, however, the inhibitory effect of the excess amount of rabbit serum became less marked, whereas with the prozoning amount of horse serum the inhibition remained.

3. With regard to the number of extracellular pneumococci it should be indicated that the figures given are not absolute, and therefore minor differences may be disregarded. One point is very significant, however. With the prozoning amount of horse serum a very rapid and almost unchecked multiplication of bacteria occurred after the first hour. This result differs little from those previously reported (2) with reference to the infectious process in the absence of immune serum. This effect is undoubtedly related to the marked inhibition of phagocytosis, as will be indicated below. It is presumed that some multiplication also occurred under the other conditions, but it is obvious that in the latter cases active phagocytosis tended to keep the number of extracellular pneumococci at relatively low levels.

4. With respect to the numbers of peritoneal white cells it will be noted that during the first 2 hours there was little difference under the various conditions. At 4 hours, however, definite changes had occurred. Thus with the large amount of horse serum the mean number of white cells per cubic millimeter of peritoneal washings was 5,340, while with the corresponding amount of rabbit serum there were 12,370. There was no marked difference between the results when the two sera were used in smaller amounts. From these results it is obvious that the large amount of horse serum inhibited the leukocytic response, whereas with the corresponding amount of rabbit serum an exaggerated response was obtained.

5. The figures with regard to the proportion of white cells active as phagocytes are obviously of comparative value only during the first hour, since in this phase of the infection the numbers of bacteria and numbers of cells were similar irrespec-

tive of the amount and species source of the immune serum. Within the earlier time periods only one fact is outstanding: large amounts of immune horse serum inhibit phagocytosis, whereas with immune rabbit serum in the same quantity the result obtained is unaltered from that with the optimal protective quantity.

6. With respect to phagocytic indices, it has previously been pointed out that this index is partly conditioned by the tightness with which the pneumococci are agglutinated. For example, with large amounts of serum one observes that the capsules are definitely more swollen than when smaller amounts of serum are employed, and consequently in the former case fewer individual bacteria are found in clumps having the same dimensions. Since immune rabbit serum produces an even greater degree of capsular swelling, it was expected that these phagocytic indices might be somewhat lower. These predictions were borne out by the results observed during the first hour, but the indices after this time are probably of little significance because of other changes which had occurred.

It would be fortunate indeed if one could take these several determined variables and from them, either directly or after suitable weighting, determine the quantitative importance of the various factors involved in the protective action of immune serum. At this time, however, an analysis of this order is not possible.

These observations permit the conclusion that the prozone in protection tests with antipneumococcus horse serum is due primarily to a marked inhibition of phagocytosis brought about by the use of an excess of serum. This inhibition is so extreme during the first 2 hours (the monocytic phase (2)) that rapid multiplication of the pneumococci occurs. Subsequently (during the polymorphonuclear phase) this inhibition is not apparent, but for the complete removal and destruction of the now enormous number of bacteria the number of white cells is insufficient, since with the large amounts of horse serum the leukocytic response is decreased. With comparably large amounts of rabbit serum there is no inhibition of phagocytic activity at any time, but the rate of removal of bacteria is somewhat low in the earliest phases of the process because of the low phagocytic index, which in turn is dependent upon the marked capsular swelling induced by rabbit serum.

Although these experiments give information concerning the manner in which the protective process is modified by prozoning amounts of immune horse serum, they offer no satisfactory answer to the question as to the primary cause of this result.

The more obvious explanation is that immune horse serum contains some substance which inhibits phagocytosis *per se*, and that this substance is not present in immune rabbit serum. It has already been reported (2) that various sera, protein solutions, etc., inhibit the phagocytosis of rough pneumococci. Experiments were therefore carried out to determine the comparative inhibitory effects of horse and rabbit serum under these conditions. It was found that both of these sera, if used in large amounts, produce a definite and almost equal inhibition of phagocytosis of rough pneumococci, but that this effect is only apparent immediately after the injection of serum and culture. By the end of the first hour the phagocytic rates in animals which had received either of the sera were quite similar to those found in animals receiving no serum. At no time did the immune horse serum cause an inhibition of phagocytosis greater than that produced by immune rabbit serum.

As a second method of study, experiments were carried out in which various heterologous materials were added to the injection mixtures of type-specific immune sera and virulent Type I pneumococci.

The results of these experiments are shown in Table II. It will be noted that with the optimal protective amount of this lot of horse serum the survival rate was 74 per cent. With four times this amount the rate was only 25 per cent. Neither egg white nor normal rabbit serum caused a significant reduction in the expected survival rate. The rate with Type II antipneumococcus horse serum was decreased. With a concentrated antimeningococcus horse serum, and with the serum of a rabbit fed with cholesterol for 10 days, a very marked decrease in the rate of survival was obtained. The contrasting results with normal and with lipemic rabbit serum are very striking.

With immune rabbit serum, on the other hand, not only does an excess amount fail to produce a prozone, but no significant inhibition of protection is obtained upon the addition of the various heterologous materials.

These findings lead to certain deductions as to the essential causation of the prozone phenomenon with antipneumococcus horse serum.

1. From the studies on the phagocytosis of rough pneumococci it is apparent that immune horse serum contains no unique heterologous inhibitory substance.

2. It is possible, however, that the prozoning effect is directly related to some property of the specific antibody or to some property of the resultant of antigen-antibody union. This possibility is strongly supported by certain experimental facts: (a) The prozone is obtained with immune horse serum and not with immune rabbit serum. (b) The prozoning effect with immune horse serum is greater the higher the antibody content (1). (c) The protective action of optimal amounts of antipneumococcus horse serum may be blocked by certain

TABLE II

Effect of Heterologous Materials on the Protective Action of Antipneumococcus Sera

Each mouse received 0.1 cc. Type I pneumococcus broth culture plus the following reagents	Antipneumococcus horse serum			Antipneumococcus rabbit serum		
	Total mice	Sur- vival	Sur- vival rate	Total mice	Sur- vival	Sur- vival rate
			<i>per cent</i>			<i>per cent</i>
0.05 cc. specific immune serum	38	28	74	18	18	100
0.2 " " " "	16	4	25	15	15	100
0.05 " " " " + 0.15 cc. egg white	17	12	71	12	12	100
0.05 cc. specific immune serum + 0.15 cc. normal rabbit serum	17	11	65	12	11	92
0.05 cc. specific immune serum + 0.15 cc. Type II antipneumococcus serum	16	8	50	12	12	100
0.05 cc. specific immune serum + 0.15 cc. concentrated antimeningococcus serum	17	4	24	12	12	100
0.05 cc. specific immune serum + 0.15 cc. lipemic rabbit serum	19	4	21	12	12	100

heterologous reagents. The contrary is true with immune rabbit serum.

3. These results support the view that the prozone is due primarily to a unique property of the horse antibody and secondarily to some heterologous component of the immune serum, possibly of a lipoidal nature. It is conceivable that the immune reaction product with antipneumococcus horse serum may have the capacity to combine with some detrimental lipid component of the serum. This conjecture is supported by chemical studies as to the lipid content of immune aggregates (3). It has been found, for example, that immune precipi-

tates formed by the union of capsular polysaccharide and antipneumococcus horse serum selectively adsorb cephalin, while those formed with immune rabbit serum fix lecithin.

That the results in protection tests with antipneumococcus horse serum should be different from those with specific immune rabbit serum is not surprising in view of their many distinctive properties.

SUMMARY

1. Type I antipneumococcus horse serum, in amounts exceeding a characteristic optimum, fails to protect mice against infection with the homologous type pneumococci. This failure is due to a marked inhibition of the phagocytic mechanism in the earlier stages of the infectious process. On the other hand, antipneumococcus rabbit serum in similar quantities does not inhibit phagocytosis, nor does it block the protection.

2. The experimental evidence suggests that the prozoning action of immune horse serum is due primarily to some characteristic property of the specific antibody and secondarily to an heterologous component of the serum, ineffective in itself but acting through the mediation of the antigen-antibody combination. This secondary factor may be a lipid.

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THE PROTECTIVE ACTION OF TYPE I ANTIPNEUMOCOCCUS SERUM IN MICE

V. THE EFFECT OF ADDED LIPIDS ON THE PROTECTIVE MECHANISM

BY KENNETH GOODNER, PH.D., AND FRANK L. HORSFALL, JR., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, May 28, 1936)

In preceding papers (1, 2) it has been reported that Type I antipneumococcus horse serum, in amounts exceeding a characteristic optimum, fails to protect mice against infection with the homologous type pneumococci. This effect was shown to be associated with a marked inhibition of the phagocytic mechanism in the earlier stages of the infectious process. Antipneumococcus rabbit serum in similar quantities does not produce this prozone phenomenon.

The available evidence (2) suggested that the prozoning action of immune horse serum is due primarily to some characteristic property of the specific antibody and secondarily to an heterologous component of the serum, ineffective in itself but acting through the mediation of the antigen-antibody combination. There was a strong suggestion that this secondary factor was lipid in character.

The present report deals with the effect of isolated and purified lipids on the protective action of antipneumococcus serum.

EXPERIMENTAL

The various technical procedures used in the protection tests and in the analysis of its mechanism have been detailed in previous papers (1-3). Type I antipneumococcus horse and rabbit sera have possessed equivalent antibody content.

Lecithin and cephalin were prepared by methods outlined in detail elsewhere (4). Chemical analyses have shown that these phosphatides possessed a high degree of purity. The cholesterol used was a commercial preparation of orthocholesterin (Schuchardt). Cholesterol oleate was obtained through the courtesy of Dr. I. H. Page. Solutions of these reagents were prepared by slightly different

methods, but the general procedure involved the preparation of saturated alcoholic solutions except in the case of cephalin where ether was employed as the solvent. Suspensions or solutions of the required strengths were made from these solutions by dilution in physiological saline. Cephalin solutions prepared in this fashion are water clear. With the other lipids, opalescent colloidal suspensions are obtained.

The amounts of lipid employed varied from 0.1 to 0.4 mg. per mouse (17 to 23 gm.). In each case this amount of lipid formed a part of a 1.0 cc. inoculum. These amounts are well within what might be called a physiological range. Average figures for the lipid content of serum may be listed as follows:

Total lipid.....	2.0-15.0 mg. per cc. of serum
Free cholesterol.....	0.2- 1.3 " " " "
Cholesterol esters.....	0.5- 5.0 " " " "
Phosphatides.....	0.3- 3.5 " " " "

The Effect of Added Lipids on the Protective Action of Antipneumococcus Horse and Rabbit Sera

Various amounts of purified lipids were added to combinations of specific immune sera and Type I pneumococci. The summarized results of these experiments are shown in Table I in terms of percentage survival. Since within this range the amount of each lipid used gave results which differed but little (with one exception), only the combined survival rates will be considered in this analysis.

With antipneumococcus horse serum lecithin produced no significant change in the expected survival rate. The addition of either cephalin, cholesterol, or cholesterol oleate, on the other hand, tended to block the protective action of the immune serum. In experiments not summarized in the protocol, it was found that crude commercial lecithin (Merck) also inhibited protection.

With antipneumococcus rabbit serum no important alteration in survival rate was obtained by the addition of any of these lipids. The lowest rate was with cephalin but the difference as against the controls is scarcely significant.

These results may be better understood in the light of certain findings with reference to the lipid content of immune aggregates (5). Thus it has been found that the lipid adsorbed upon the immune precipitate formed on the interaction of antipneumococcus horse serum and capsular polysaccharide consists of cholesterol, cholesterol esters, and *cephalin*. That adsorbed upon immune rabbit serum precipi-

tates contains similar amounts of the cholesterol, but the phosphatide is *lecithin*. In a sense, then, one may say that the reaction of the capsular polysaccharide with the horse antibody fixes cephalin, while that with the rabbit antibody fixes lecithin. This occurs not only under ordinary conditions, but also upon the addition of purified lipids to the reactive mixtures. Moreover, as has been pointed out by other workers, lecithin and cholesterol are antagonistic in an immunological sense (literature on lipid antagonisms reviewed by

TABLE I

Effect of Added Lipids on the Protective Action of Type I Antipneumococcus Sera

Immune serum	Added lipid	No lipid added	Amount of added lipid			Mean survival rate irrespective of amount of lipid added
			0.4 mg.	0.2 mg.	0.1 mg.	
Antipneumococcus horse serum	None	71				71
	Lecithin		69	80	87	78
	Cephalin		13	13	33	20
	Cholesterol		30	21	44	35
	Cholesterol oleate		0	38	88	42
Antipneumococcus rabbit serum	None	96				96
	Lecithin		100	90	90	93
	Cephalin		86	85	80	83
	Cholesterol		94	89	89	91
	Cholesterol oleate		100	100	100	100

Figures indicate survival rates. Average of fifteen mice for each combination of lipid and immune serum. Each mouse received 0.1 cc. Type I pneumococcus broth culture, 0.05 cc. immune serum, and indicated amount of lipid.

Degkwitz (6)). In the light of these observations, and assuming that both cephalin and cholesterol tend to block protection, it is possible that the lipids adsorbed in the case of rabbit reactions are not inhibitory because the lecithin tends to offset the action of the cholesterol. The addition of cephalin is without effect because it is not fixed. In the case of antipneumococcus horse serum all of the adsorbed lipids are inhibitory to protection, hence the addition of either cephalin or cholesterol tends to reduce the protective action.

In addition to the foregoing experiments others were undertaken in which combinations of lipids were added to protection tests set up

with antipneumococcus horse serum. The results of one such experiment are shown in Table II.

It will be noted that while the addition of lecithin did not alter the general survival rate both cephalin and cholesterol tended to block the protective action of the immune serum. On the other hand, when cholesterol and lecithin were added together the blocking action of the added cholesterol was eliminated. No significant degree of protection was obtained in either combination in which cephalin was present.

These results indicate that with combinations of lipids, lecithin offsets the effect of the added cholesterol. Since it is known that lecithin is not fixed by the union of capsular polysaccharide and the

TABLE II

The Effect of Combinations of Lipids on the Protective Action of Type I Antipneumococcus Horse Serum

	No. of mice	No. surviving	Survival rate
			<i>per cent</i>
Controls without lipid.....	50	36	72
Lecithin added.....	10	8	80
Cephalin added.....	10	2	20
Cholesterol added.....	8	2	25
Lecithin and cephalin added.....	8	1	13
Lecithin and cholesterol added.....	8	7	88
Cholesterol and cephalin added.....	8	2	25

In each instance 0.05 cc. of immune serum, 0.1 cc. of culture, and 0.2 mg. of the respective lipid were injected.

antibody of immune horse serum, it must be assumed that the added lecithin has produced this effect in some other manner. In chemical studies it was found that the addition of lecithin to a reacting mixture of immune horse serum and specific capsular polysaccharide greatly diminishes the amount of cholesterol adsorbed on the immune precipitate. In these combinations, therefore, the action of the lecithin is an indirect one, since it simply tends to block the fixation of the added inhibitor, cholesterol.

These results indicate that the lipids affect protection through their participation in the antigen-antibody reaction either directly or indirectly. It seems possible that the adsorbed lipids may affect the

properties of the sensitized pneumococci in such a manner as to render them less readily phagocyted, and thereby inhibit the protective action to be expected from the immune serum.

In order to approach this possibility directly, studies were carried out upon the rate of phagocytosis of sensitized pneumococci as influenced by lipids. The results of these experiments are shown in Table III. Each figure represents the average of determinations on three or more mice.

From these results it will be noted that with immune horse serum both cephalin and cholesterol caused a marked inhibition of phagocy-

TABLE III

Effect of Added Lipids on the Rate of Phagocytosis of Sensitized Pneumococci

Each mouse received 0.1 cc. Type 1 pneumococcus broth culture plus the following reagents	Cells active as phagocytes during 1st hr.	Pneumococci intracellular		
		1 hr.	2 hrs.	4 hrs.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.05 cc. immune horse serum.....	11	39	36	97
" " " " + 0.4 mg. cholesterol....	3	2	13	51
" " " " + " " lecithin.....	9	30	22	87
" " " " + " " cephalin.....	2	10	8	67
" " " rabbit "	13	40	69	100
" " " " + 0.4 mg. cholesterol....	14	7	64	98
" " " " + " " lecithin.....	10	21	48	96
" " " " + " " cephalin.....	10	9	30	88

tosis as judged not only by the number of cells active as phagocytes but also by the intracellular disposition of the pneumococci. With immune rabbit serum neither of these lipids produced a significant alteration in the phagocytic rates.

DISCUSSION

These findings demonstrate that added lipids affect the protective action of antipneumococcus serum by direct or indirect participation in the process of specific sensitization of the microorganism by the antibody of immune serum. Thus the result depends not only upon the character of the particular lipid but upon the properties of the

antigen-antibody complex as well. It has been found by chemical studies that when the antipneumococcus antibody from the horse combines with the specific capsular polysaccharide, there occurs an adsorption of cholesterol and *cephalin*, but that with the antibody from the rabbit, cholesterol and *lecithin* are adsorbed. In the present paper it has been shown that the addition of cholesterol and of cephalin blocks the protective action of antipneumococcus horse serum but does not materially affect the protective action of antipneumococcus rabbit serum. In the latter instance cephalin is not effective, since it does not enter into the reaction. The negative effect of the cholesterol is undoubtedly offset by the antagonistic action of lecithin which does participate.

These findings have a direct bearing on a second problem. Amounts of horse serum, in excess of a definite optimum, fail to protect mice. This phenomenon, which has been termed the protective prozone, is not obtained with antipneumococcus rabbit serum. In previous work it was shown that this prozone effect was in a measure conditioned by the nature of the antibody and also partly by some secondary component of the immune serum, possibly lipid in nature. The present results offer an explanation for the prozone phenomenon, for it is obvious that increasing the volume of serum not only gives an added amount of antibody, but also increases the lipid available for adsorption. The fact that the prozone phenomenon occurs with the immune serum of one species and not with that from another is explicable on the basis of selective lipid adsorption.

SUMMARY

1. The addition of small amounts of cholesterol and of cephalin reduces markedly the protective action of antipneumococcus horse serum.
2. These lipids do not affect the protective action of antipneumococcus rabbit serum.
3. These findings may be explained (*a*) by the selective adsorption of lipid on the antigen-antibody complex, and (*b*) by certain lipid antagonisms.
4. The failure of large amounts of immune horse serum to protect mice against pneumococcus infection is explicable on the basis of

selective participation of lipids dependent upon the species from which the antibody is derived.

5. The lipids modify the results of protection tests only through participation in the process of specific sensitization.

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OBSERVATIONS ON THE RELATION OF THE VIRUS CAUSING RABBIT PAPILLOMAS TO THE CANCERS DERIVING THEREFROM

I. THE INFLUENCE OF THE HOST SPECIES AND OF THE PATHOGENIC ACTIVITY AND CONCENTRATION OF THE VIRUS

By PEYTON ROUS, M.D., JOHN G. KIDD, M.D., AND J. W. BEARD, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 24 TO 26

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The carcinomas deriving from the papillomas induced with a virus in domestic rabbits (1) have this virus as their primary cause. The present paper is concerned with the carcinogenic effect of various strains of the virus,—meaning thereby the infective materials procured from the “spontaneous” papillomas of different cottontail rabbits,—and with the influence of the concentration of the inoculum, and the method whereby it is introduced. In corollary certain unusual tumors will be described which have followed upon the papillomatosis engendered by the virus in a cottontail rabbit.

Virus Concentration as Affecting Cancer Incidence

Many domestic rabbits have been inoculated into scarified skin areas of standard size with serial dilutions of virus or with serum mixtures in which it had been partially neutralized incidentally to titration tests (2). Some of these animals have been retained for months, until malignancy developed. The records made, of the time of appearance and situation of the cancers, show that the more concentrated the inoculum the sooner and oftener, in general, did cancer appear.

The method of inoculation has already been detailed. The various materials were rubbed into scarified squares about 4 cm. across, situated on the belly and lower sides of the test animals. The emerging growths ranged from solitary or scattered papillomas, punctate

in origin, to masses of confluent papillomatosis occupying the entire square. All concentrations of virus beyond a certain minimum gave rise forthwith to growths of the latter sort, these appearing at about the same time, looking alike, and behaving in the same way. Inocula containing slightly less virus than the minimum mentioned caused crowded, discrete papillomas which became confluent masses after a few days, a negligible interval when compared with the many months that often elapsed before cancer appears at even the most favorable site. During the long precancerous period, the scattered or solitary papillomas produced by still more dilute virus frequently enlarged to a diameter of several centimeters or united to form a broad mass of precisely the same appearance as if the area had been papillomatous from the beginning; and throughout this period all of the growths of any one rabbit behaved in the same way with rare exceptions, all enlarging, becoming stationary, or retrogressing together. It was to have been expected that the larger the area involved primarily in papillomatosis the more often in general would cancer occur, and needless to say this was the case. But a further fact presented itself: Cancer appeared soonest and most often, generally speaking, in those areas of confluent papillomatosis which had resulted from the most concentrated inoculum,—this although other areas had been covered with confluent growths of precisely similar aspect, for the same length of time. Some illustrative instances follow:

1. A "departiculated" (3) 25 per cent extract of virus W. R. 1240 was prepared (4 cc. of Tyrode to every gram of glycerinated cottontail papilloma), a portion was diluted to 10 per cent, and part of this was passed through a Berkefeld filter V, with further dilution of the filtered and unfiltered materials in multiples of 10. All the fluids were then inoculated into squares on domestic rabbit K 9-5 (Table I). The symbols employed to record the character of the growths that arose as result of the inoculations were: + —, one or two discrete papillomas; +, a small number of discrete papillomas; ++, many discrete papillomas; + + +, semiconfluent papillomatosis; + + + +, confluent papillomatosis. Tracings were made of the growths at intervals of a few days or weeks, but the system of plus marks employed in the tables gives a better index of how they were constituted. They often increased in size greatly, but their number did not do so after the first few weeks. Most of the records made during the long precancerous period have been omitted from the tables.

The cancers that developed from the papillomas were ulcerated, fungoid or depressed, invasive and destructive tumors, arising in the papilloma. All of them

progressed, most metastasized, and they regularly led to death of the animal. The histological findings confirmed the clinical diagnosis.

Table I shows that where the three most concentrated materials had been inoculated the growths were primarily confluent, that by the 47th day those from several of the other inocula had become semiconfluent, and that at the 96th day five confluent masses of approximately the same size were present, with two others, semiconfluent and somewhat smaller. Thereafter all retained the same general aspect and relative proportions until the 7th month when ulcerating, invasive cancers appeared in the two masses due to the most concentrated inocula.

2. Rabbit 3-74 (Table II) was inoculated with mixtures of depariculated virus W. R. 632 and the partially neutralizing sera of papillomatous rabbits. A control mixture with Tyrode solution was also employed in duplicate. The latter gave rise promptly to diffuse papillomatosis, whereas the most pathogenic of the serum mixtures produced growths that remained semiconfluent during many weeks. After some months, however, this difference disappeared, and when all the masses were measured on the 186th day, six of them had practically the same size and an identical appearance. By the 247th day ulcerative, spreading cancers had appeared in two of them, namely those deriving from the inocula which had given rise forthwith to confluent growths. These were the control mixtures with Tyrode. By the 275th day another cancer had established itself, as result of the inoculum in which the virus had undergone least neutralization.

3. D. R. 4-91 (Table III). Mixtures were made of virus suspension with serum specimens taken from three normal jack rabbits (*Lepus californicus*, Gray) and an animal (rabbit IV) of this species which had a "spontaneous" papilloma (4). This last specimen neutralized the virus almost completely, while those from the other individuals proved somewhat less favorable to it than the Tyrode of a control mixture. By the 41st day, however, the growths from four of the inocula were confluent, and from that time on their aspect was identical. Cancer did not put in an appearance until the 300th day, and then only where the virus-Tyrode mixture had been introduced.

4. D. R. 3-16 (Table IV) was inoculated with graded dilutions of virus 632. The growths produced by the 1 per cent and 10 per cent virus were luxuriant from the first, and indistinguishable in their character, but those from the lower dilutions appeared later and never reached the same size. By the 428th day cancer had appeared in the mass due to 1 per cent virus, and by the 447th day in that due to the 10 per cent preparation.

Additional instances of the phenomenon here illustrated could be provided were this necessary. In two of the tabulated experiments the virus was not diluted with Tyrode but instead was partially neutralized with immune serum. This has the same effect as dilution: it acts to cut down the number of effective virus entities, not to alter their pathogenic capabilities (2). The tables show that the

Cancer Incidence as Determined by Virus Concentration

TABLE I. Test rabbit D. R. K 9-5, inoculated with graded dilutions in Tyrode of virus W. R. 1240.

Virus strength	24 days	47 days	96 days	201 days
25%	++++	++++	++++	++++C
10% " filtered	++++-	++++	++++	++++C
1%	++++	++++	++++	++++-
" "	++-	++++	++++	++++
0 1%	+	++-	++++	++++
" "	+	++++	++++	++++
0 01%	0	+-	+	+

TABLE II. Test rabbit D. R. 3-74, inoculated with virus W. R. 6-32 mixed with Tyrode and with the sera of papillomatous domestic rabbits.

Virus	Inocula		Rabbit No.	24 days	41 days	186 days	247 days	275 days
	Tyrode	Serum				cm.	cm.	
6 3/8%, 0.5 cc.	cc.	cc.	-	++++	++++	4 x 3	4 x 3	++++C
	0.5	-	-	++++	++++	4 x 3	3 x 3	++++C
	"	-	V	++++	++++	4 x 2.5	4 x 2.5	++++C
	0.3	0.2	"	++++	++++	5 x 2.5	4 x 2	++++
	-	0.5	VI	++++	++++	4 x 2	4 x 2	++++
	0.3	0.2	"	++-	++-	4 x 1.5	6 x 2	++
	-	0.5	VII	++	++	3 x 3	3 x 3	++++
	0.3	0.2	"	0	+-	1.5 x 1	1.8 x 1.8	+-
	-	0.5	VIII	0	+-	2 x 1.5	1.5 x 1.5	+-
	0.3	0.2	-	++-	++	3.5 x 2	3.5 x 1	++++
2 3/8%, 0.5 cc.	0.5	-	-	++-	++			++++

TABLE III. Test rabbit D. R. 4-91, inoculated with virus mixed with Tyrode and with the sera of normal and papillomatous jack rabbits.

Virus	Inocula		Rabbit No.	14 days	19 days	26 days	41 days	300 days
	Tyrode	Serum						
6%, 0.5 cc.	cc.	cc.	—	++—	++++—	+++++	++++	++++—C
	0.5	—	I	++	++++	+++++	++++	+++++
	0.3	0.2	II	++	+++	+++++	++++	++++
	"	"	III	++	+++	+++++	++++	++++
	"	"	IV	0	0	0	++	++
	—	0.5	I	++	++	+++++	++++	++++
1%, 0.5 cc.	—	"	II	++	++++	+++++	++++	++++
	—	"	III	++	++++	+++++	++++	++++
	0.5	—	—	++	+++	++	++	++
	—	0.5	I	0	0	+	++	0
	—	—	II	0	0	0	++	0
	—	—	III	0	++	+	+	+

TABLE IV. Test rabbit D. R. 3-16, inoculated with graded dilutions of virus W. R. 6-32.

Virus strength	per cent	16 days	24 days	45 days	211 days	428 days	447 days
					cm.	cm.	
10	+	+++	++++	++++	++	++++	++++C
1	+	+++	++++	++++	++	++++C	++++C
0.1	0	0	+	+++	++	++	++
0.01	0	0	0	++	++	++	++
0.001	0	0	0	++	++	++	++
0.0001	0	0	0	0	0	0	0

0 = negative. + — = 1 or 2 papillomas. + = 3 to 8 papillomas. ++ = many scattered discrete papillomas.
 + + + = semiconfluent areas of papillomatous proliferation. + + + + = confluent papillomatous proliferation covering inoculation site.
 C = cancer present.

concentration of virus employed to produce confluent papillomatosis notably influenced the incidence of the cancers. The reasons for this become plain when one considers the composition of the virus-induced growths.

Composition of the Papilloma as Determined by Virus Concentration

The papillomas result from the multiplication of those cells which become infected with the virus at the time of inoculation, no discernible involvement of neighboring elements taking place. It follows that the growths are inevitably multicentric in origin and composite in character, save in those presumptive instances in which the initial infection has involved but one cell. Often the appearance of the solid growths resulting from broadcast inoculation of scarified areas gives no hint of their composite nature. But in not infrequent cases portions of the mass are gray with pigment while others are pink (Fig. 1). The virus infection frequently discloses the existence of local differences in the skin that are imperceptible while it is normal, the young, multicentric growth being spotted or stippled in various shades of gray (Fig. 2). If the centers of proliferation are numerous and crowded, the growth may assume a pepper and salt aspect, or be evenly gray, a color which darkens as the projecting mass keratinizes and dries. The diversity of hue is due to the inclusion of stimulated melanoblasts in certain of the proliferating cell aggregates, as not in others.

Successive tracings of confluent papillomas, notably of those assuming this form secondarily, have disclosed the fact that many of the clefts separating them into peaks, or larger subdivisions, are the boundary lines between all hosts of differing lineage, deriving from different cell-virus associations, that is to say. These clefts may persist for many months. Often after a time cell families of greater vigor than their neighbors proliferate at the expense of the latter, pressing these to one side, perhaps extending more deeply into the connective tissue (Fig. 3) and forming usually discrete, squat domes or onion-shaped growths (Fig. 4). These are often nonpigmented or only slightly so, in contrast to the rest of the mass, and they are notably liable to cancerous change, as we have often observed, a matter which will be discussed further on. To what their proliferative vigor is due,

whether to the influence of exceptionally active or numerous virus entities, or to a special susceptibility of the cells primarily infected, or to some unusual suitability of virus entity and individual cell, cannot at this writing be said. Whatever the reason, it is plain that some cell-virus associations are especially favorable to proliferation. The more concentrated the virus introduced into a given expanse of scarified skin the greater should be the number of such associations; and the greater in consequence the likelihood of cancer.

In another way the virus concentration may influence cancer incidence, namely by bringing about a crowded condition within the proliferating mass. Where competing cell families are most numerous crowding will be most pronounced, the epithelium most disordered, the supporting connective tissue most disturbed, and bacterial infection most frequent—conditions all that further malignant change (1).

An important inference for experimentation follows from the observations as given: If one wishes to obtain cancers as soon as possible after inoculation one should introduce concentrated virus into considerable skin areas. Malignancy will ensue only after months of proliferation at the earliest and generally where the papilloma is oldest. Growths that have arisen by enlargement from one small inoculated spot contain but a small proportion of old papillomatous tissue as compared with others of the same size that have been confluent from the beginning. It is no accident that cancers appearing in growths of the first sort are usually situated near their center; for here the papilloma is oldest, and favoring local disturbances have been present longest.

The Carcinogenic Activity of Virus Materials of Differing Derivation

The more vigorously the papillomas grow the sooner and oftener does cancer occur. Recognizing this we have ordinarily discarded those virus materials which were prone to produce retrogressing or indolent papillomas. In comparing the carcinogenic potentialities of the more pathogenic strains, not only must the method of inoculation and the concentration of virus be taken into account, but many other influences. The papillomas of Dutch belted rabbits grow especially well, and hence they are more favorable to malignancy than agouti rabbits; the general character of the skin of animals of the same breed

has some influence; and so too has the local skin character, cancer developing much more frequently in black haired regions than in white ones of the same individual. Certain interferences with the papilloma precipitate the malignant change (1), and to these we have often resorted. Because of these complicating factors and the variety of our experiments, it is difficult to analyze them as a whole. This much is clear from them however, that every virus material which gave rise to papillomas that grew progressively in several animals for many months proved carcinogenic in one or more of them, irrespective of the animal breed and of whether the inoculation had been into scarified areas or by tattooing into a number of small spots. Chart 1 records the incidence and time of appearance of cancer after inoculation of the four virus materials most extensively used. They were all inoculated as 3 per cent or 10 per cent extracts of glycerinated papilloma tissue. To narrow the conditions of comparison, only those animals are entered in the chart which were of agouti or Dutch breed, inoculated in one of the two ways just mentioned, and as result carrying papillomas which had grown progressively during the first 3 months, without later tendency to retrogress. This entailed the omission of a large proportion of the data.

Cancer never asserted itself clinically before the 4th month after inoculation, at earliest after 132 days, in an animal not charted because it had been inoculated with a strain of virus (W. R. 18) employed in a very few rabbits. Of the viruses figuring in the chart, W. R. 538+638 had the shortest incubation period, giving rise to papillomas within 7 to 14 days, and W. R. 632 the longest period, 11 to 25 days, W. R. 738 and W. R. 1240 occupying intermediate places (papillomas after 10 to 19 days). In most instances the growths produced with W. R. 538+638 were progressive, whereas many caused by W. R. 632 disappeared after a few weeks and a large proportion of the others retrogressed subsequently. This material was extensively used because of its suitability for experiments on the antiviral power of the blood. Retrogression was considerably less frequent with strains W. R. 738 and 1240.

When Chart 1 is scrutinized it will be seen that the more active the virus material, as evidenced by incubation period and subsequent course of the papillomas, the earlier did cancer appear. The fre-

quency of malignancy varied directly with the vigor of the growths, in accordance with our previous experience, but on this point the chart provides no information. Several materials have proved carcinogenic besides those tabulated. Virus W. R. 18 which gave rise to the earliest cancer thus far noted was as actively papillogenic as virus 538+638, if not more so. Virus 738 still caused cancer after passage through a snowshoe rabbit (*Lepus americanus*, Erxleben).

Virus strain	Incubation period (days)	Retrogressions	Months elapsed												
			4	5	6	7	8	9	10	11	12	13	14	15	
W.R. 538+638	7-14	Few	○△ ○○	▽ ○	●● ○	●● ○	●▽ ○	▽▽							
W.R. 1240	10-18	Moderate No.			● ○○	▲ ○○	▲▲ ▲▲ ▲▲ ▲▲								
W.R. 738	10-18	Moderate No.					▲ ○○	○		○ ○○	○○	○			
W.R. 632	11-25	Many	○ ○○	▽ ○○	○○ ○○	○○ ○○	● ○○	● ○○	▽		● ○○	●● ○○	●●	▲ △	

Breed Agouti Dutch

Scarified ○ ●

Tattooed △ ▽

Black symbols indicate cancer

CHART 1. Virus strain and cancer incidence. Each symbol stands for the growth in one rabbit, and only those rabbits have been considered in which the papillomas grew well. A black symbol means the occurrence of cancer in the month designated, and a white symbol that no malignancy had appeared in the existing papillomatous tissue to the time of record, which was either that of last examination or of death.

The Incidence of Cancers in Cottontail Rabbits

From one to three rabbits of every shipment of 24 coming from our source of supply in southern Kansas has had somewhere on its skin a virus-induced papilloma or several of them; and this although the dealer has endeavored to retain all animals carrying such growths. He traps many thousand rabbits yearly, and it is to his interest to send us those having ulcerated lumps in association with papillomas, but of the several thus far received none has proved cancerous, nor has

Shope encountered any in the several hundred cottontails he has utilized. Malignancy is evidently a rare event, but it does occur, for Syverton and Berry have described an instance (5), and we have now noted one. Theirs was a metastasizing squamous cell carcinoma first noted developing from a "spontaneous" growth 85 days after the cottontail came under observation.

In our laboratory five cottontails with notably vigorous growths have been maintained for periods of 14 to 23 months and four more for from 9 to 11 months. The growths were due in most instances to carcinogenic virus strains. Of the three animals surviving 23 months, one ultimately developed both a carcinoma and a metastasizing sarcoma. It had received especially active virus.

W. R. 26 was inoculated by the tattooing method with 5 per cent virus 538+638 at two spots 2 mm. across near the tip of each ear. Scharlach R in olive oil was injected beneath one of each pair of the discrete growths that soon appeared, and this was done twice again at intervals of a few weeks. The growths thus stimulated became somewhat the larger, and remained so later. By the 8th month all were enormous, radish-shaped cutaneous horns, tough and black, with turgid, darkly pigmented, fleshy bases (Fig. 5), and such they continued to be throughout almost another year. Then the horny masses began to flake away (Figs. 6 and 7), the pigmentation of their discrete, slaty bases was gradually replaced by a purply pink hue, and discrete protuberances of rounded or football shape, ranging up to 1.4 cm. in diameter, formed at several places on the tense margins of the growths on one of the ears (Fig. 6). Biopsy of such a protuberance yielded the picture of an orderly, cellular fibroma, with almost no mitoses; and during the next 2 months they all dried into brown, nut-like masses as if their circulation had been cut off. Meantime the cutaneous horns became irregularly striated, they dried down to the ear level and largely flaked away, and at some points a narrow, raised, very firm ring of new tissue appeared about them, separated from them by a narrow cleft. The ring was largest about the biggest horn, which was now buff-colored and apparently calcified in its basal portion, with a slight thickening and scabbing directly beneath it on the under side of the ear. Biopsy showed the ring to consist of keloid-like tissue with cancellous bone in its midst. During the ensuing and final month, a discrete nodule, apparently of the same keloid tissue, about 3 mm. across and 2 mm. high, developed some 6 mm. away from the largest horn on the other ear, toward the base. The animal died after 683 days in all. At this time nearly all of the horny material had recently come away, leaving flat ulcers. The microscope showed beneath these on both ears, slightly thickening them, an unencapsulated spindle cell sarcoma, which in some regions contained neoplastic giant cells. It had penetrated to the inner side of one ear, occupying the scabbed region already mentioned as opposite the buffy,

dead papilloma, but it had caused only a slight protrusion there. The discrete, small nodule near the horn on the other ear consisted of sarcomatous tissue. There were numerous small metastases replacing but not greatly enlarging the lymph glands at the base of both ears, a larger metastasis 6 mm. across in a salivary gland adjacent to one of them (Fig. 11) and numerous secondary growths up to 4 mm. across in the lungs. At all these situations the growth was a simple, spindle cell sarcoma. Remarkably little living papilloma persisted where it had grown vigorously for so long; but at one of its original sites where a proliferating small remnant of it was still present an indubitable squamous cell carcinoma (Fig. 10) had almost entirely replaced it. The cancer was only about 1 cm. across, very shallow,—nowhere more than 2 mm. thick,—and it was situated well away from the sarcoma. Its cells were not joined to those of the papilloma, but had engulfed some of the melanotic pigment of the latter where they were destroying it, and at one point they had penetrated into the new-formed cancellous bone.

In this instance repeated biopsies had failed to disclose the existence of either the sarcoma or the carcinoma, and these growths gave so little indication of their character during life that they were lost for experimental purposes. Furthermore they had caused such local destruction that it was no longer possible to tell where they had arisen. This was notably true of the sarcoma: one cannot even be certain whether its presence in both ears was consequent on metastasis from a primary situation in one of them. During the long precancerous period the papillomas obviously acted as local irritants, notably where calcification had taken place, and both keloid and bone formation resulted. That the sarcoma was another result of the irritation seems likely; and it is conceivable that the carcinoma may have originated in this way as well, from epidermal cells adjacent to the papilloma but not themselves infected with the virus. The histological findings did not suggest carcinoma sarcomatodes.

Two snowshoe rabbits with papillomas consequent on virus inoculation into scarified areas have been under observation for more than a year, with no malignant change as yet. Judging from the behavior of the growths, the animals are only moderately favorable to the virus.

DISCUSSION

The differences found in the carcinogenic activity of the virus materials can all be referred to differences in their ability to produce and maintain actively proliferating papillomas of the usual sort. It

seems probable that any strain of the Shope virus capable of giving rise to vigorous growths in domestic rabbits will prove carcinogenic through their agency.

The similarity of the growths produced by the various inocula was remarkable. There was no morphological indication of any qualitative difference in their pathogenicity, much less that any of them contained virus entities which were distinctively carcinogenic. Yet the wide range in their carcinogenic effect depended on more than a mere numerical difference in their content of papilloma-producing entities. Some of the virus materials provided entities which gave rise individually to vigorous growths, and others only such as caused indolent, retrogressing ones,—a fact very evident in the behavior of the papillomas of punctate origin resulting from the inoculation of high dilutions of virus into broad areas of skin. All of the cottontails supplying the materials came from a region of the Southwest in which the papillomatosis is endemic (Kansas, Iowa, Oklahoma, Texas). The size of this region, the existence of the disease there for a very long period (it has been known for more than 50 years), and its chronicity in the individual rabbit would lead one to look for virus variants. But certainly none of major calibre has been encountered thus far in the study of the “spontaneous” growths or those purposely induced with the virus.

The composite character of the virus-induced papillomas has already been discussed to some extent in its bearing upon carcinogenesis. The fact that most of the growths consist essentially of aggregates of competing cells, deriving from a greater or less number of primary cell-virus relationships, must be kept in mind during any consideration of how the cancers come about. It provides yet another reason, besides those already recorded, for the local origin of the malignancy. As already remarked, some of the cell-virus relationships formed upon broadcast inoculation of a skin area are more favorable than others to an eventual cancerous change. Why this should be the case is not yet clear.¹

¹ In this connection the fact deserves mention that a variety of tumors, including cancers, originate from the epidermal cells of tarred skin after the virus is brought into association with them by intravenous injection (Rous, Peyton, and Kidd, J. G., *Science*, 1936, 83, 468).

The dominant cell aggregates from which cancer arises tend to be nonpigmented or to become so before it occurs, a fact difficult to reconcile with the observation that the incidence of cancer is greatest in the papillomas produced on pigmented skin and themselves pigmented. Yet the same holds true of the tar cancers, these occurring oftenest on pigmented skin, though themselves nonpigmented or soon becoming devoid of pigment (6).

The infrequency with which cancer develops from the papillomas of cottontail rabbits cannot be laid to any peculiarities of their fabric which tend to prevent malignancy in general, for carcinoma can be readily induced by tarring their skin. In three of nine cottontails with ears tarred three times a week for 11 to 15 months,² squamous cell cancers have occurred on both ears at nearly the same time in each case, metastasizing to the lungs in one of them, to a lymph gland at the base of one ear in another, and in all proving so destructive locally as to force amputation of the organs for conservation of the material. None of a more considerable group of domestic rabbits tarred in the same way for a much longer period with result in large papillomas has developed an indubitable, progressive cancer.

An obvious reason for the failure of virus-induced papillomas to become cancerous in cottontails is to be found in the lack of vigor of the growths, a feature generally attended by a negative outcome in domestic rabbits. Cottontails are notably resistant to the virus despite the fact that it maintains an endemic disease in them. From 1 to 3 out of every 10 cottontails experimentally inoculated with active virus prove insusceptible, and in more than half of the others retrogression of the induced growths sooner or later occurs, though they are often vigorous to begin with. In the few remaining animals papillomatosis persists until death, that is to say for periods up to nearly 2 years, in our experience. After the growths have become established they remain stationary in size for many months as a rule, though still proliferating. We have yet to observe papillomas in a cottontail that grew so large as to prove fatal, a frequent happening in domestic rabbits. The virus and the wild rabbit are evidently habituated to each other, as is often the case when the association between a parasite

² Horizontal retort tar of the Oster-Gasfabrik of Amsterdam, kindly provided by Dr. Karl Landsteiner.

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and its host species has endured for a long time. According to Theobald Smith symbiosis is the eventual result of such a state of affairs (7). The ability of the Shope virus to lie latent over long periods in cottontails (8), as not in domestic rabbits, seems worth recalling in this connection.

SUMMARY

All the strains of the Shope virus thus far tested which give rise to vigorous, progressively enlarging papillomas in domestic rabbits, function as carcinogenic agents by way of these growths. The more pathogenic the virus as evidenced by the brevity of its incubation period and the vigor of the papillomas produced, the sooner and oftener does cancer occur. The number of virus entities contained in the inoculum notably influences the outcome, cancer appearing most frequently in those confluent, papillomatous masses which have resulted from the greatest concentration of the virus material under test. The papillomas experimentally induced by the ordinary inoculation methods are essentially aggregates of proliferating cell families, each the outcome of some primary cell-virus association. Some of these associations are followed more frequently by cancer than others are in the same animal.

Cottontail rabbits, the natural hosts of the virus, are notably resistant to its sustained activity, as compared with domestic rabbits. Though often growing rapidly at first, the papillomas of cottontails soon become relatively inert in most cases, and they usually retrogress, and rarely undergo malignant change. In an instance here reported both a squamous cell carcinoma and a metastasizing sarcoma appeared at the base of some papillomas due to experimental inoculation, which had existed on the ears of a cottontail for nearly 2 years.

The meaning of the phenomena is discussed.

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EXPLANATION OF PLATES

PLATE 24

FIG. 1. Particolored papillomatous expanse resulting from a broadcast inoculation of Shope virus into the scarified skin of the abdomen of a brown-gray domestic rabbit (21st day). Considerable areas of the growth are gray or black, while others are nonpigmented. The normal skin had given no indication of the differences now apparent. $\times\frac{1}{2}$.

FIG. 2. Pigmented and nonpigmented, proliferating cell aggregates consequent on the inoculation of various dilutions of virus into the skin of a domestic rabbit (27th day). Where the aggregates are numerous and crowded, as in the confluent mass on the right of the photograph,—which resulted from the least dilute material,—they are difficult to discern individually. $\times\frac{3}{4}$.

FIG. 3. Side view of a papillomatous expanse produced by the inoculation of a wild rabbit (90th day). The growth has been raised with the aid of the finger and a slide in order to show better a discrete, nonpigmented nodule which has proliferated with especial vigor and extended below the general level. The rest of the mass is sooty, but reflections from its surface, notably in the region beneath the edge of the slide, tend to confuse the picture. $\times\frac{3}{4}$.

FIG. 4. Papillomatous masses consequent on tattooing the virus material into two small spots on the side of the same rabbit (90th day). One mass consists of numerous dark gray peaks, all of about the same height, and the other contains some similar ones of various sizes, but all these latter have been pushed aside by a rapidly enlarging, almost nonpigmented, dome-shaped growth. $\times\frac{3}{4}$.

FIG. 5. Cutaneous horns (8th month) due to tattooing the Shope virus into two spots on each ear of cottontail W. R. 45. The larger growth on each ear had been injected with Scharlach R some weeks previously. $\times\frac{1}{2}$.

PLATE 25

FIGS. 6 and 7. Same growths as in Fig. 5. The dry cutaneous horns have mostly come away (20th month). Fig. 6 shows the rounded fleshy protuberances that had formed at the border of the remaining low papillomas, and Fig. 7 the calcification of one of the horns on the other ear (arrow).

FIG. 8. Growth of Fig. 6 (22nd month). The protuberances have dried down, puckering and folding the ear. Only the tip can be seen of one of them (A), which is separated from the other by a furry strip (seen vertically in Fig. 6). A ring of firm tissue has formed around the base of the nearer growth, and a few milli-

meters away is an intracutaneous nodule (B). Both were found to be sarcomatous at autopsy.

FIG. 9. Remains of the calcified horn of Fig. 7 with a thickening next its base (arrow), which proved to be sarcomatous.

PLATE 26

FIG. 10. Section of the thickening of Fig. 9. Some new-formed bone can be seen at the center of the picture, with the intact epidermis above. A squamous cell carcinoma is invading the deep tissue. $\times 90$.

FIG. 11. A metastasis from the sarcoma of Figs. 9 and 10. It has replaced the lymph node at the base of one of the ears and invaded the adjacent salivary gland. $\times 90$.



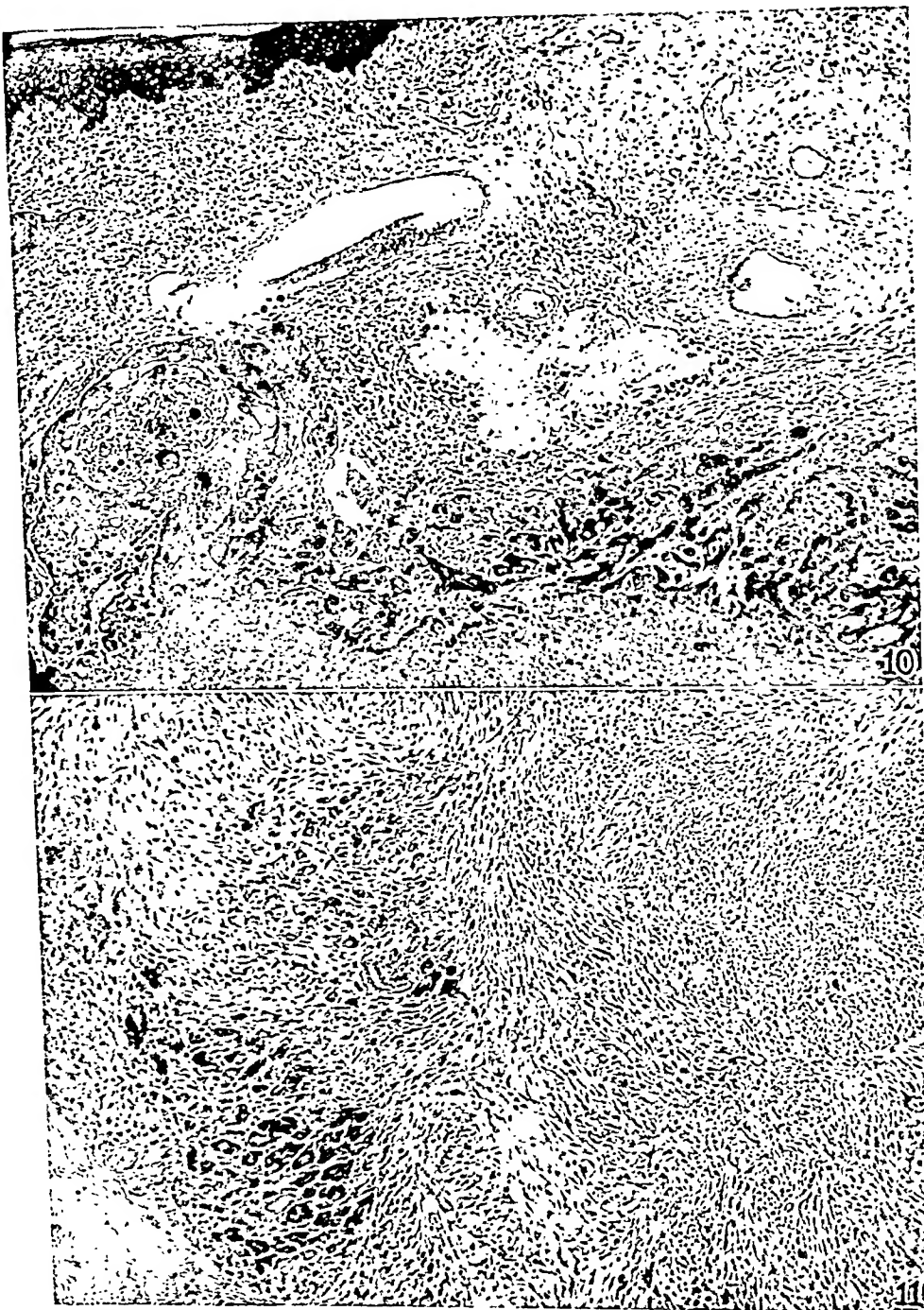
Photographed by Joseph B. Haulenbeek

(Rous *et al.*: Cancers derived from papilloma virus. I)



Photographed by Joseph B. Haulenbeek

(Rous *et al.*: Cancers derived from papilloma virus. I)



Photographed by Louis Schmidt

(Rous *et al.*: Cancers derived from papilloma virus. I)

OBSERVATIONS ON THE RELATION OF THE VIRUS CAUSING RABBIT PAPILLOMAS TO THE CANCERS DERIVING THEREFROM

II. THE EVIDENCE PROVIDED BY THE TUMORS: GENERAL CONSIDERATIONS

BY PEYTON ROUS, M.D., J. W. BEARD, M.D., AND JOHN G. KIDD, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 27 TO 33

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In an accompanying paper the relationship of the Shope virus engendering papillomas to the cancers deriving from these growths has been approached from the side of the virus. It will now be considered in the light of certain data provided by the tumors themselves.

The papilloma is known to be characterized by those traits of appearance and behavior which distinguish neoplasms from other pathological processes (1). Much new evidence on this point has lately come to hand.

The Subcutaneous Extension of Papillomas

The papillomas produced experimentally in domestic rabbits, by the inoculation of virus into scarified areas of skin, not infrequently invade the layer of reactive tissue that gradually forms beneath them, with result in deep pearls lined by the virus-infected epithelium. An instance has been figured in a previous paper (1). When papillomas are prevented from growing outwards by covering them with a layer of collodion soon after they appear they push down until checked by the fibrous corium and form subepidermal masses that may become large (1). Three instances have been observed in which, as result of essentially similar pressure conditions, occurring naturally, the "spontaneous" papillomas of the cottontails burrowed into the subcutaneous tissue. The dealer had been requested to send us all rabbits carrying lumps under or next the papilloma.

W. R. 40 N, from Kansas, had on receipt a rounded, discrete mass in the subcutaneous tissue of the inner surface of the right thigh (Fig. 1). It was cream-colored, an irregular, somewhat flattened sphere, 4.5 by 4.5 by 3.0 cm. across, of doughy consistency, with a sooty, mat-like skin papilloma, 1 cm. high and 2 by 1.5 cm. in diameter, surmounting and attached to it. The surface growth was so situated as to be constantly pressed upon when the animal sat crouched, and it had a flat, worn top. About 3 cm. away, where the skin covering the most projecting portion of the deep mass had been stretched tense, it had undergone ischemic necrosis and separation over an area about 1.5 cm. across. When the scab thus formed was pulled away there was disclosed a cyst filled with irregularly concentric lamellae, each about $\frac{1}{2}$ mm. thick, composed of a slightly moist, soap-like material which shelled out clean save at one spot at a considerable distance from the surface mass, where a nonpigmented papillomatous growth, on a base about 1.5 cm. in diameter, projected inwards from the cyst wall. The lamellae,—which had been formed by keratinization of the epithelium of the growth just mentioned,—were ranged in layers about it like the petals of a cabbage rose. Elsewhere the cyst wall was smooth. Its contents were readily shelled out and the deep papilloma was brought to the surface by pushing it through the hole left by the scab, where it retained its position, proliferating as a cream-colored, vertically striated cylinder which on biopsy had the usual, papillomatous character. In consequence of the new state of affairs the sooty, surface papilloma was turned at right angles to its former position, and, thus relieved of pressure, it built up rapidly into a high cone (Fig. 2).

Two other cottontails were found to have subcutaneous masses nearly as large as that described and capped like it with mat-like papillomas exhibiting pressure effects. One of the surface growths was situated on the lower abdomen where the thigh pressed upon it, the other just back of the axilla. In the latter instance the low, dry papillomatous peaks were worn down into a cup, where the upper joint of the fore leg pressed. Elsewhere in the case of this animal, there were several jagged, superficial papillomatous expanses of the ordinary sort on distant skin regions free from pressure. The surface papillomas were sooty in both rabbits, whereas the deep growths were creamy. In the regions where these projected most the skin had undergone a dry necrosis.

All three rabbits are still under observation.

In several instances of skin papillomas experimentally induced in cottontails deep extensions of aggressive character have developed secondarily. Two of these will be reported upon.

A 5 per cent extract of glycerinated virus material, W. R. 595 + 596, a notably active material, was rubbed into a scarified square on the right side of a cottontail (5 N). A solid mass of characteristic sooty papillomatosis developed, which soon became a high, truncated cone that occupied the entire area of inoculation

and was dry, vertically ribbed and almost black. Its living, slowly advancing, bulging, basal edge was sooty in hue practically everywhere. After 10 weeks, knobs were felt on the under side of the cone, well within its edge, and small rounded spheres, discrete or partly coalesced, had appeared outside (Fig. 3). They were situated in the subcutaneous tissue and could be moved in all directions save away from the main growth, palpation disclosing that each was connected with this by a narrow, flexible cord. Two stages in the later course of events are shown in Figs. 4 and 5. The outlying nodules, almost spherical when first noted, soon elongated, and the distance between them and the main growth gradually widened, as result of growth at their outer side combined with resorption at the inner. With one exception the nodules that traveled away most rapidly from the surface mass had rounded, creamy outer ends, as seen through the skin; but further back, the nodule was ruddy with distended vessels, and so too was the overlying skin. In the exception mentioned the growth advanced by thrusting out prong-like processes from a broad, deep extension of the surface mass, and these became thickened and somewhat blunted (Fig. 6). As time passed the cords connecting the distant nodules with the primary growth could no longer be felt, but their disappearance became progressively less complete as the nodules crept further away, owing to the continual enlargement of the latter, a process which obviously involved the leaving behind of more and more keratinized material.

The migrating nodules followed the course of the large veins in all cases, and their advance was furthest in the general direction of the axilla, though one of them curved aside after a time as if it had met an obstruction (Fig. 5). They are still advancing and enlarging. All are creamy in contrast to the melanotic, cutaneous papilloma. The latter has been lifted several centimeters above the skin level by a mass of deep growth which has gradually formed under it (Fig. 6). This is firmly attached to it, and has the shape of partially fused spheres.

In order to learn the method of advance of the growth the two prong-like extensions were excised, together with the tissue about them, and sectioned serially, one in its long axis, the other transversely through its tip. The excision was done on the 138th day, when the prongs were advancing but slowly. Like the subcutaneous masses previously studied, they consisted mostly of keratinized epidermal cells (Fig. 10) which underwent constant additions through the activities of a thick, living layer of epithelium which had the invasive character manifested by the virus-induced papilloma when growing after experimental implantation in notably favorable situations within the body (1). The main direction of advance was along and between some large blood vessels. In sagittal section the prong was wedge-shaped, but the outline of the wedge was scalloped because the epithelium had directly invaded the underlying voluntary muscle at numerous situations, the invading tongues later broadening and keratinizing (Fig. 27). Only by the secondary keratinization with cyst formation could the growth be discriminated from a squamous cell carcinoma. It was markedly desmoplastic, surrounded everywhere by new, reactive connective tissue, save in a few places where its

advance appeared to be taking place with such rapidity (Fig. 27) that it had outstripped the reactive proliferation round about.

The growth had progressed between vein and artery, where were the large lymphatics as transverse sections have shown. At the furthest point reached, however, the epithelium had ceased to proliferate, and rounded out into small cysts enclosed in a thick layer of connective tissue wherein the lymphatics were lost (Fig. 7). The main direction of its advance had been checked, as in the case of one of the large nodules of Figs. 4 and 5, which first followed the vessels and then curved sideways. At numerous situations elsewhere it was actively invading, though, and indeed in some places penetrating directly between the muscle fibres (Fig. 27).

While the findings clearly indicated that the subcutaneous extensions had taken place along the lymphatics, if not within them, it was plain that the growth could advance independently of these channels, by direct invasion of the tissues. In the following instance, of a less aggressive papilloma, the penetration along the lymphatics was especially noteworthy.

A 5 per cent extract of virus material W. R. 18, which is especially active, was tattooed into a number of scarified spots on the sides of W. R. 45. Characteristic discrete horns developed, reaching a diameter of 1 to 1½ cm. by the 55th day, but thereafter not enlarging greatly. Owing to oversight the animal was not examined again until the 134th day. Then six smooth, spherical or oblong nodules from 0.8 to 1.5 cm. in diameter were present in the subcutaneous tissue of the right side, under or near the skin papillomas, four of them completely independent of these latter. A tough, narrow cord could be felt connecting a fifth with a surface papilloma, and the sixth lay beneath one of the latter and was fixed to it.

In the subcutaneous tissues of the left side, under and attached to several of the tattoo papillomas, was a large mass rather like a lobulated kidney in shape (Fig. 8), 6 by 4 by 2 cm. in diameter, with several attached spheres or pendule-like protrusions; and there were two other deep nodules further off, one 1 cm. away from the nearest surface growth, the other 5 cm. away and in the precise position of the superficial inguinal gland. The nearer of these two seemed wholly independent of all of the other growths, but the further, which was bean-shaped and 1.5 cm. in longest diameter, was connected with the main subcutaneous mass by a rounded strand 0.15 cm. thick. It was removed for section together with the adjacent portion of this strand, and a few weeks later a "pendule" was also taken for section (Fig. 11). Both proved to be cysts walled with papillomatous epithelium of the characteristic sort and filled with keratinized scales formed by the proliferation. The strand connecting the main, deep mass with the inguinal growth,—which showed no remnant of lymph gland,—consisted of connective tissue undergoing fibrosis, with dying or dead epithelial cells along its center (Fig.

of other opportunity, and then laterally in consequence of pressure conditions, until they had emerged from beneath the horn. None sprang directly from the edge of the latter, where expansive growth could take place. The deep extensions must have been narrow and cord-like on first emerging from beneath the horn, for they did not attract attention then; but with the later relief from pressure they rounded out at their further end into cysts.

The papillomas experimentally produced in domestic rabbits by inoculation with the Shope virus are far more formidable growths than those in wild rabbits, and they much more frequently go on to cancer; yet they do not penetrate into the subcutis though frequently pushing down during the precancerous period into the reactive tissue immediately beneath them, or extending laterally just beneath the epidermis with result in superficial, outlying pearls. They are checked by the stout, fibrous layer of the corium, and in consequence their proliferation is confined to the loose connective tissue immediately beneath the epidermis, unless indeed the fibrous barrier has been weakened by inflammatory changes, in which case they may go somewhat deeper. In wild rabbits no such barrier exists, the corium being a flimsy, web-like structure.

The Survival and Proliferation of Embolic Fragments of the Papilloma

The virus-induced papillomas frequently penetrate into the blood and lymph vessels but their cells adhere to one another, retaining the tenacious association that is so evident in the high, peaked surface growths. Instances of unaided metastasis formation have yet to be observed, but slight operative interferences are followed not infrequently by the development of secondary nodules in the lungs.

D. R. 4-06, an agouti rabbit employed for neutralization tests, developed papillomatosis on seven squares of skin where mixtures with virus 6-32 and serum or Tyrode had been rubbed in. After 10 weeks Scharlach R was injected into one papillomatous mass, extracts of dried and glycerinated cancer tissue into two others, and a string impregnated with a Tyrode extract of a cancer was threaded through a fourth mass and left there. No cancers ever developed; but when the animal died, after 311 days in all, a sphere 1 cm. across, consisting entirely of dead papillomatous tissue, was found in a lung (Fig. 9). The surface growths were now indolent.

13). All of the deep nodules were creamy, in contrast to the sooty hue of the surface growths, except one which appeared to be of a dubious, pale gray as viewed *in situ*.

The skin over those subcutaneous masses which projected most dried and came away soon after they were first observed, with loss of some of the surface papillomas and of the soap-like contents of the cysts. The exposed epithelial lining of the largest of these now underwent a surface proliferation forming papillomatous peaks like those already present, save that the new ones were cream-colored. Other cysts became infected with pus-producing bacteria and eventually were resorbed *in toto*. A few small ones which had not ruptured persisted for many months as such. At death, on the 630th day, only one remained, a somewhat flattened cyst of the ordinary sort. Its epithelium showed mitoses and in one region was directly invading the reactive connective tissue.

As in the case of W. R. 5 N, the subcutaneous extensions advanced farthest in the direction followed by the main lymphatic channels. There is every reason to suppose that the nodule in the inguinal region had replaced a lymph gland. The general course of events was like that Handley has reported for cancers of the human breast (2); and the eventual death and resorption of the epithelial cord (Fig. 13) connecting the distant nodule with the main mass was such as he has described. But despite these features, and the direct invasion of the muscle that took place in cottontail 5 N, the papilloma remained merely such, at worst a locally malignant growth.

It will have been noted that with one dubious exception all of the subcutaneous extensions in cottontails were devoid of pigmentation, whereas the skin papillomas were invariably sooty. One may suppose that under the conditions of downgrowth the stimulated melanophores, passively included in the proliferating surface mass (3), were left behind. But another reason existed for the creamy hue. The downgrowths evidently derived from the most vigorously proliferating cells in the papillomas,—which were themselves essentially composites of competing cell aggregates (4),—and such aggressive aggregates usually become non-pigmented in time, even when growing on the skin surface.

In the case of W. R. 5 N, the surface papilloma was spotted with dark gray during the early days of its formation, plain evidence of its composite character. When it had become a solid, horn-like structure, the cell aggregates well within its base were inevitably pent on all sides save beneath; and downwards the more vigorous ones grew in default

of other opportunity, and then laterally in consequence of pressure conditions, until they had emerged from beneath the horn. None sprang directly from the edge of the latter, where expansive growth could take place. The deep extensions must have been narrow and cord-like on first emerging from beneath the horn, for they did not attract attention then; but with the later relief from pressure they rounded out at their further end into cysts.

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of the emboli were undergoing degeneration after having done well primarily and become attached to the vessel wall. Their epithelium was dying and the material at their center had separated as if from fluid accumulation (Fig. 19).

None of the emboli that consisted of only a few cells had given rise to a growth: each of the developing nodules had at its center a piece of the surface papilloma that was of relatively large size. The new-formed connective tissue attaching the embolic masses to the vessel wall had frequently become covered by endothelium, but this had undergone no further proliferation, and the neighboring alveolar epithelium had altered no further than to assume the cuboidal form where the pressure of the developing growth had caused atelectasis.

The findings left no doubt that the lung growths arose solely by the proliferation of cell emboli. Schmidt has reported, in a paper now classical (5) that the lung metastases of visceral carcinomas arise from the larger cell emboli only, and that these become attached to the arteriolar wall by new connective tissue extending into the pulmonary tissue. Where such attachment fails to take place the embolus may live for some time, its cells often forming a syncytium; yet though in immediate contact with the vessel wall they fail to penetrate it.

The occurrences leading to death or establishment of the papilloma cells were precisely like those noted by Schmidt. Yet this parallelism in the course of events cannot be taken to mean more than that the fate of pulmonary emboli composed of epithelial cells capable of proliferation and invasion is determined by conditions and reactions that are characteristic of their situation.

Factitious Malignancy

When small pieces of a virus-induced papilloma, procured soon after it appears, are implanted in the voluntary muscles of the host, growths usually develop promptly, and, as already remarked, they may look and act as if malignant when they are proliferating in the midst of connective tissue proliferation called forth by bacterial infection. Yet they do not metastasize even when they have the aspect of squamous cell carcinomas (as determined by biopsy), and later they may retrogress if the infection is conquered by the host, their epithelium first reverting to the orderly papillomatous form, and eventually keratinizing entirely,—changes clearly recorded in the markings of the dead material. The influence of local infection to determine the fate of papillomatous nodules developing from cell emboli that reach the

node, however, and the growths in the legs, were progressing actively, but all these had the aid of local inflammatory processes. The insusceptibility of the lung tissue to infection by the Shope virus, the ease with which the papilloma can be transplanted to the interior of the host, and the circumstances under which the pulmonary nodules developed in the foregoing instances (and in others previously reported) point to cell emboli as responsible for them. It has seemed well nevertheless, to carry out an experiment whereby the fate of such emboli could be directly ascertained.

Two brown-gray rabbits were inoculated into scarified abdominal areas with a 5 per cent extract of virus material W. R. 538 + 638, and confluent papillomatosis soon appeared. A few days later some of the growth was shaved away, cut fine, suspended in Tyrode solution, and injected into an ear vein. After another 5 days the animals were killed. The lungs showed scattered, minute, translucent, gray, rounded, dot-like solidifications with, in the case of one rabbit, a few slightly larger dots of the same sort, up to 1 mm. in diameter. Numerous blocks were taken and sectioned serially.

The lung findings were the same in both animals. Many small, discrete foci of characteristic papillomatous growth were present. At the center of even the largest of these the remains of an embolus could be discerned, often to be identified as such by fragments of hair included in it (Fig. 16). Many dead or dying emboli were also present, lodged in arterioles, usually where they forked. The cells of some of these had proliferated before they succumbed, and formed syncytia (Fig. 17). Remarkably little fibrin had collected about them, but a few scattered polymorphonuclear leukocytes were present in the neighboring tissue, as generally happens where the squamous epithelium of tumors is dying.

The findings were especially informative in serial sections that happened to parallel the arteriole, so that it could be seen both proximal and distal to the cell embolus (Fig. 18). All of the latter had some platelet clot about them, and those that were proliferating had lost their angular form and rounded out by a lateral proliferation of the surface epithelium (which showed frequent mitoses), so that they had come to consist of a core of dead, keratinized material and a living rind. The arteriole was distended by their enlargement, and sometimes the neighboring bronchiole had undergone a sabre-sheath compression. The best established emboli had become more or less surrounded by new-formed connective tissue and fixed upon the arterial wall. Where this had happened, and only here, the proliferating epithelium had extended into the lung, pushing through the new connective tissue as a carcinoma does (Fig. 19), penetrating the stretched and thinned media of the vessel, and spreading in the loose pulmonary tissue, with compression or occupation of the alveoli (Fig. 16). Around some of the advancing growths there was a sparse scattering of lymphocytes, and some

of the emboli were undergoing degeneration after having done well primarily and become attached to the vessel wall. Their epithelium was dying and the material at their center had separated as if from fluid accumulation (Fig. 19).

None of the emboli that consisted of only a few cells had given rise to a growth: each of the developing nodules had at its center a piece of the surface papilloma that was of relatively large size. The new-formed connective tissue attaching the embolic masses to the vessel wall had frequently become covered by endothelium, but this had undergone no further proliferation, and the neighboring alveolar epithelium had altered no further than to assume the cuboidal form where the pressure of the developing growth had caused atelectasis.

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lungs has been illustrated by the case of D. R. 4-78 (Fig. 12). It is not possible to tell whether the spontaneous invasion of voluntary muscle by the deep extension from the surface papilloma of W. R. 5 N (Fig. 27) was induced by local infection, though the profusion of reactive connective tissue about the growth suggests such a possibility. The papillomas exhibiting factitious malignancy of the sort now under discussion have a feature in common which distinguishes them from the anaplastic carcinomas arising from the papilloma, though not always from the other growths that originate from the latter: they keratinize secondarily in an orderly way and form cysts filled with lamellated material. None of the various stimuli that we have brought to bear on the papilloma has caused it to become enduringly anaplastic like the more malignant of the squamous cell cancers that eventually develop from it.

The Tumors Derived from the Papilloma

A not inconsiderable variety of tumors derive from the papilloma. As already reported, all are the outcome of changes in the epidermal cells infected with the virus, and all are expressive of progress in a single direction, namely toward anaplastic squamous cell carcinoma-tosis (6). The study of a large material has confirmed and extended these findings.

Many of the derivative tumors differ but little from the parent growth, some being recognizable only by their encroachments upon it and others by slight histological peculiarities in addition. Even those closely resembling the ordinary papilloma exceed it in aggressiveness: otherwise they would not attract attention. In our previous papers instances have been pictured which illustrate these facts. Discrete "onions" of aggressive, atypical proliferation may appear in the midst of a papillomatous mat, or "papillomas of the second order,"—so called because they have an unusually complicated pattern (Fig. 20),—or invasive cystic tumors. Occasional aggressive growths have been encountered which resemble ordinary, virus-induced papillomas so absolutely in their morphology that their status has remained uncertain (Fig. 21). When the alteration toward malignancy has been but slight the tumor may not become irreversible in its course. The growth like a glans penis that progressively replaced the ordinary

papilloma of D. R. 2-48 (6) never invaded the underlying tissue, and during the months before death took place it became stalked and much smaller, owing in some part to maceration of its surface. Its course was reminiscent of that of many tar papillomas. In general, however any considerable alteration toward anaplasia has been attended both by irreversibility and the tendency to metastasize. We have yet to observe a manifest, squamous cell carcinoma which did not pursue a progressive, destructive course. The cancers of D. R. 2-38, which appeared to be retrogressing when this animal was last reported upon (6) brought about death in the end and had merely become scirrhus.

Especial interest attaches to those tumors of problematic malignancy which have metastasized; for sometimes the secondary nodules differed in no essential respect from such as result from the experimental implantation of ordinary papillomatous tissue in favorable sites (Figs. 22, 23, 28, 29). More will be said upon this head further on. As might be expected retrograde local changes and necrosis are frequent in consequence of compromised vessels, and some cell nests may perish, owing to complete keratinization. A squamous cell metastasis in the lungs became almost entirely necrotic as result of pressure conditions that cut off its blood supply.

The new tumors that are expressive of but slight changes in the papilloma seldom grow large. We have never observed a "papilloma of the second order," for example, that was more than 1 or 2 cm. in diameter, nor has metastasis occurred from a growth of this sort. Such tumors tend to undergo further change in the direction of squamous cell carcinomatosis, as do the others that are relatively benign; and their enlargement is so slow as to provide ample opportunity for the change to supervene. The much more rapidly growing, malignant cystic papillomas (6) often kill the host before anaplastic changes have become advanced, though they are usually perceptible here or there. Such growths extend under the skin, forming subepidermal pearls and involving it in ulceration (Fig. 30), and they advance rapidly toward the axilla or groin along the course of the lymphatics (Fig. 31), and frequently metastasize to the regional glands (Fig. 24). The malignant papillomas which are not cystic very soon change into squamous cell carcinomas, but the papillomatous character is occasionally evident in the metastases, which are usually confined to the local glands

(Figs. 25 and 26). Squamous cell carcinomas almost regularly metastasize, both locally and often to the lungs as well, the frequency of the latter happening corresponding in general with their degree of anaplasia.

A noteworthy tendency exists for multiple cancers of the same individual to be all of one general sort, though frequent exceptions are observed. They may be mostly fungoid, malignant papillomas, cystic papillomas or eroding, desmoplastic, squamous cell carcinomas. The longer the host survives the greater is the likelihood that all will be of this last type, however they may have begun. Those animals which are relatively unfavorable to cancer, as evidenced by its late appearance, are especially the ones in which it assumes and long retains the cystic form.

While cancer is the most obvious consequence of the changes in the papilloma, the other tumors deserve equal, if not greater attention. All are expressive of the potentialities of the cells originally infected with the virus, a limitation which does not exclude considerable diversity of type, as has been seen. Multiple tumors of a single kind, occurring in the same animal, may differ slightly yet distinctively.

Induced Malignancy

It is possible to precipitate malignancy by various stimulative interferences with the papillomas. Many instances attesting to this fact have been procured since our first publication (6); and injection of Scharlach R into the base of papillomas has been repeatedly utilized to bring on cancer before its time. The success of this procedure is the more remarkable because the dye is not itself a carcinogenic agent, a fact sufficiently attested by the outcome of its clinical use. Bün-geler (7) has reported the development of cancer after 15 months in a rabbit repeatedly injected with the dye; but there was an associated arsenical keratosis. The epithelium of papillomas caused by the Shope virus responds far more actively to Scharlach R than does ordinary epidermis, and under its influence large fungating and burrowing cystic growths rapidly form; yet if stimulation is stopped retrogression or reversion to ordinary papillomatosis may ensue, even after the dye injections have been repeated at intervals during many weeks.

It is our experience that for a long time, 3 months at the least,

the virus-induced papillomas continue to be merely such no matter how stimulated, whether by transplantation, incision, vaccinia or bacterial infection, Scharlach R injections, or the permanent insertion of strings impregnated with dry or glycerinated cancer material,—procedures all that have been repeatedly utilized.

On the assumption that removal of the papilloma cells to an especially favorable milieu might disclose the existence of malignancy sooner than would otherwise be the case, we have operated repeatedly on the same papilloma at intervals of a few weeks, on each occasion implanting bits of it in the muscles of one of the legs of the host. Each time the material was taken from the same spot at the base of the growth, a procedure possible because it regenerated rapidly in the intervals. Thus cells were procured that had the additional stimulus incident to repair. But though the experiment was carried out upon several domestic rabbits with vigorously growing papillomas, no positive results were obtained. True, some of the leg nodules did eventually become cancerous, as proven by extension through the fascia, involvement of the skin, and metastasis formation, but this happened only after the lapse of several months when the surface growths had also undergone malignant changes.

The Transition to Cancer

As already reported, the development of malignancy is preceded by alterations in the aspect and behavior of the papilloma. Its enhanced vigor of growth, loss of pigmentation, tense, fleshy base, the crowded disorder of its cells, their extension downward and laterally to form “extramural” pearls, and an increasing irregularity of keratinization (6), all bespeak progress toward malignancy. Slight interferences may now precipitate this, yet often they do not, however frequently repeated, and some growths may remain merely more active and aggressive papillomas, long after neighboring ones in the same host have become cancers. The multiple papillomas produced in any one animal by tattooing virus into the skin all behave alike with occasional noteworthy exceptions (8), and all may enlarge rapidly at one period, then perhaps become stationary or dwindle, and after a while enlarge again. The observation that the more vigorously the papillomas proliferate the more likely is cancer to ensue has been abundantly confirmed; yet not infrequently it appears in some one of a number of shallow based, unpromising growths,—doubtless as a result of local stimulative occurrences. Carcinosis is obviously an outcome of

favorable conditions which may be very local, and operative during a brief period only. It is conceivable that, when they happen to be notably favorable cancer may arise even in a papilloma that is retrogressing everywhere else; but the phenomenon has still to be encountered.

Morphological Evidence of the Influence of the Virus on the Cancers

A large proportion of the tumors deriving from the papillomas exhibit some of the histological characters of the latter.

In producing papillomas, the virus acts primarily on the germinal layer of the epidermis: its cells enlarge, and often become tall and narrow, and the nucleus also enlarges, though it retains the vesicular form. Rapid multiplication by mitosis forces most of the cells toward the surface, and since proliferation still goes on in the polygonal layer this becomes unnaturally thick. The granular layer consists of elements less flattened than normal, more coarsely and irregularly granulated, and frequently containing parakeratotic lumps that are sometimes large. The cells die before they have finished flattening and keratinizing, and they heap up into tenacious, dry, scoriaceous or horny peaks.¹ The same series of events, comprising a pathological differentiation, usually takes place in the nodules resulting from implantation of the growth within the host, the cells preserving their organoid association and manifesting the usual tendency to form papillae with narrow supporting cores, though now these papillae point toward the center of the mass instead of outwards, and the keratinized material is soft.

In all save the most anaplastic of the tumors deriving from the papillomas,—those composed of elements that no longer differentiate to any noteworthy degree,—the sequence of changes just described can be more or less clearly perceived.

The fact has already been stressed that there are growths of which it is impossible to decide whether they are merely ordinary papillomas of unusually aggressive habit or new and different tumors (Fig. 21). The cystic papillomas have precisely the aspect assumed by the ordinary papilloma when growing after implantation in especially favorable sites, as e.g. in the leg muscles; and their metastases may be indistinguishable from the cystic nodules that result from such implantation (Figs. 22, 23, 28, and 29). The frankly malignant papillomas may give rise to secondary growths in which the papillomatous structure is retained (Figs. 25 and 26). Cystic extension along the lymphatics, such as we have encountered,¹ For further details Hurst's excellent description can be consulted (*J. Exp. Med.*, 1933, 58, 607).

incidence and character of the tumors arising from these growths. The evidence concerning the etiological rôle of the virus will be briefly reviewed incidentally to a consideration of its significance.

The virus functions as the first cause of the cancers, through the papillomas that it induces. The more active it is, as evidenced by the time of appearance and behavior of these growths, and the greater the concentration of the inoculum, the sooner and oftener does malignancy develop. The cancers originate from the cells that are proliferating under the influence of the virus. But similar facts hold true of other carcinogenic agents,—dibenzanthracene for example,—though the virus is far more effective than any of these in bringing cancer about.

The papillomas caused by the virus have the immediate character of neoplasms, and, even in cottontails, which are relatively resistant hosts, they may exhibit some of the traits associated with malignancy, —as when they grow down spontaneously into the subcutaneous tissue, invade voluntary muscle, and advance along the lymphatics after the fashion of cancers of the human breast. In domestic rabbits, unaccustomed hosts for the virus and very favorable to its action, the papillomas often verge on the malignant. Sometimes a decision as to whether they have attained this state or have merely responded to fortuitous, intercurrent influences by simulating malignancy can be made only in the light of the final happenings. The presence of the Shope virus has been demonstrated indirectly, by serological test, in two animals to which a cancer deriving from a papilloma had been transplanted; but this finding does not necessarily mean that it was immediately responsible for the cancers, since wholly extraneous viruses can persist within neoplasms and undergo transfer with them. More significant is the evidence that the virus continues to exert an influence on a large proportion of the tumors originating from the papillomas.

The facts bespeak a relation of the virus to the cancers far closer than that noted of any other carcinogenic agents thus far studied. The latter bring about a chronic tissue disturbance upon which cancer develops as an essentially different pathological process; and no evidence exists that they do more than dispose to the neoplastic state. Dibenzanthracene, the only one of them that has been followed adequately, disappears from the tumors to which it gives rise (10). The

virus on the other hand directly engenders growths of neoplastic character in which it persists, increasing in amount (11); and from these growths a variety of other tumors arise, cancers amongst them, by changes which are gradual and often slight, to all appearances.

Active strains of the Shope virus are almost unconditioned in their ability to produce papillomas. They cause a frankly infectious disease, endemic in western cottontail rabbits and notably prevalent. But the cancers which are an ultimate consequence of this disease, as produced experimentally in domestic rabbits, appear only when a conjunction of favoring circumstances has come to pass. At least four sets of influences, in addition to the primary disposition of the host and the character of the virus material, act to condition the occurrence of malignancy, namely (*a*) local characters of the skin, (*b*) the peculiarities of the individual cell-virus associations, as expressed in terms of the cellular manifestations that are their outcome, (*c*) the influences of host origin but of undetermined character, which cause all the papillomas of any one animal to wax or wane together, and (*d*) those local factors which may encourage or prevent the cancerous change. Doubtless yet other influences affecting the carcinogenesis will come to light. Cottontail rabbits, the natural hosts of the virus, ordinarily become unfavorable to the papillomatous growths after a while, as manifested by their course; and cancer is rare in this species, though it does occur. In the more susceptible domestic species, the virus, though causing, accompanying, and increasing in the papillomas, is ordinarily rendered incapable of transmitting the growth. How this happens is not yet understood. But whatever the reason it in most cases wholly removes the papillomas of domestic rabbits, and the eventual cancers as well, from the sphere of the ordinary infectious diseases. The possible influence of conditioning factors to account for the statistical incidence of cancer in human and other communities has been stressed in a previous paper (1).

Is the virus, or some variant upon it, the immediate cause of the cancers deriving from the papilloma? This question should not be approached without due recognition of certain phenomena which act to confuse the issue. Prime amongst these is the occurrence of spurious cancers. The sensitiveness of the papilloma cells to external stimulation far exceeds that of normal epidermal elements. Even the latter

can mimic cancer temporarily, as when influenced by Scharlach R; and the cells infected with the Shope virus will do this in response to numerous influences as *e.g.* those provided by the intramuscular or visceral situation, by infection with bacteria that induce a proliferative connective tissue reaction, by repeated injections of Scharlach R, etc. The reversion to ordinary papillomatosis, which often takes place later, shows that the induced malignant activities, though real enough in their effects, are not an intrinsic character of the growths.

No matter how greatly the papillomas are stimulated experimentally, they do not assume the aspect of anaplastic carcinomas; yet nevertheless a possibility exists that some of the manifestations of growths of the latter sort are due to intercurrent stimulation. They are practically always infected with pyogenic bacteria, and it is reasonable to assume that stimulation from this source or another may make as greatly for anaplastic changes as in the case of the far better stabilized papillomas. The influence of bacteria to enhance the malignancy of human tumors has long been recognized. Yet one cannot suppose anaplastic squamous cell carcinomatosis in either rabbits or men to be maintained by agents that act as adjuvants to the essential cause of the neoplastic condition. The confusing influence of such agents must be reckoned with, however.

In studying the way in which the individual rabbit cancers arise, all phenomena referable to intrusive, accidental processes must be excluded if possible; and from what has been said it is clear that this cannot always be done. The only sound course is to view the process of carcinogenesis in the large. When this is attempted one perceives that the cancers derive from the papilloma by changes which appear to be continuous, and that they often undergo further alterations until a state of marked anaplasia has been reached (6). Histological examinations at an early stage in the malignancy regularly disclose a graded morphological transition to cancer. But it must be kept in mind that pictures as strongly indicative of such a transition are not infrequently encountered where a skin cancer has united with the epidermis secondarily. Neither such findings, nor the difficulty in telling whether a papilloma or cystic growth is malignant, nor the occurrence of individual tumors which exemplify every stage in the march to anaplastic squamous cell carcinomatosis can be adduced as

proof that tumors of the latter sort result from an intensification or gradually changed activity of the virus causing the papilloma. When discontinuous variations are available in sufficiently large number they can be so arranged as to produce a specious impression of continuity. It would be possible for example to assemble arrow heads in a series which might be taken as indicating that big ones had arisen from little ones. The tumors now under consideration could also be arranged in this way. But while many of them are not cancers at all, some differing remarkably little from the parent, virus-induced growth, yet in the aggregate they are the expression of a new state of affairs. They result from local modifications of the papilloma which are not accomplished until after many weeks or months of proliferation, no matter how much the growth is stimulated. Once the preliminary period has passed, new tumors can often be readily elicited by stimuli previously powerless to bring this about; and whereas previously the neoplastic process varied but little in type, the epidermal cells multiplying and differentiating in a remarkably constant way, however great the multitude of hosts, now from the growths of a single animal a variety of tumors may arise. It is not the individual character of these tumors but their unprecedented, relatively abrupt occurrence and their diversity that constitute the significant phenomena. By and large the changes represent something more than a mere enhancement of the papillomatosis.

Recent experiments have shown that when the Shope virus is brought into association with the disordered epidermal cells of tarred skin it gives rise forthwith not to ordinary virus-induced papillomas only but to tumors of all the kinds deriving from these, including the most anaplastic cancers (12). Whatever the meaning of the phenomenon it serves to emphasize the fact that the circumstances under which the Shope virus acts are far different from those obtaining in the case of any other viruses thus far studied, save those causing the chicken tumors. The papillomas are the outcome of an enduring partnership of cell and virus, and a change in either partner may react upon the other. The activities of the virus find expression in terms of the growths it causes, but their proliferating cells provide its milieu. Any considerable changes in the virus should find prompt reflection in the morphology and behavior of the papillomas, and, on the other hand,

any disturbance of the cells may alter the environment of the virus. When the latter has been introduced into domestic rabbits this environment, though strange, proves very favorable. It is doubtless stable enough under ordinary conditions; but when the papilloma has been subjected to repeated trauma, to bacterial infection or other intercurrent influences its cells may very well undergo sufficient alteration to affect the milieu upon which the virus depends. All in all the circumstances are precisely those which should lead to a formation of variant strains of the virus, as the general experience with other viruses attests.

SUMMARY

The papillomas caused by the Shope virus sometimes grow down spontaneously into the subcutaneous tissue and extend along the lymphatics in the same way as do many cancers of the human breast. They may even invade the voluntary muscle under such circumstances, taking on an aspect suggestive of squamous cell carcinoma-tosis, but ultimately they differentiate in the way characteristic of the papilloma. Slight operative interferences with papillomas may be followed by a development of secondary nodules in the lungs. These result from cell emboli, and the same local conditions determine their fate as are effective in the case of emboli composed of human cancer cells. The virus-induced papilloma is not only a neoplasm in its immediate aspect and habit but sometimes one that verges upon malignancy. The tumors, including the cancers, which eventually derive from it in favorable hosts, are representative of more than a mere enhancement of the activity of the growth. They develop within a relatively brief period of time but only after the papilloma has grown for a long while; and they are morphologically various whereas the parent tumor is remarkably constant in its form. Some of the new growths differ but little from the papilloma, however, even when possessed of the ability to metastasize, and many continue to be influenced by the virus.

The Shope virus is heavily conditioned in its carcinogenic activity, yet it is the nearest cause for cancer now known.

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EXPLANATION OF PLATES

The sections were stained with eosin and methylene blue except where otherwise noted.

PLATE 27

FIG. 1. Subcutaneous extension of a "spontaneous" papilloma of a cottontail rabbit (W. R. 40 N). The sooty surface growth on the inside of the thigh is flat and discoid, in contrast to the irregularly spherical, cream-colored, deep mass. The skin over the latter has dried and separated in one region (arrow). $\times \frac{1}{2}$.

FIG. 2. The same growths 21 days later. The keratinized material constituting most of the subcutaneous mass had been evacuated at time of the previous photograph, and the deep lying, nonpigmented papilloma (arrow) from which it had derived was brought to the surface. This growth is now covered with dried exudate. The sooty papilloma, relieved from pressure, has heaped up into a cone. $\times \frac{3}{4}$.

FIG. 3. Beginning subcutaneous extensions from the base of a virus-induced papilloma in a cottontail (W. R. 5 N) (73 days). $\times \frac{3}{4}$.

FIG. 4. A further stage of the extension (92 days). The discrete nodules have migrated along the large veins. The apparent connections with the primary growth are due in the main to foldings of the skin, which was stretched for photographic purposes. The cutaneous horn is raised on the side toward the abdomen by a subcutaneous mass having the form of partially coalesced spheres. $\times \frac{3}{4}$.

FIG. 5. A still later stage (137 days). The direction of growth of one nodule (arrow A) has altered, and in consequence it now curves away from the vein. At

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FIG. 5. A still later stage (137 days). The direction of growth of one nodule (arrow A) has altered, and in consequence it now curves away from the vein. At

B and C are two prong-like subcutaneous extensions. These are more plainly to be seen in Fig. 6. $\times \frac{3}{4}$.

FIG. 6. Lateral view of the growth (130 days). At A is the subcutaneous nodule designated with this letter in Fig. 5, and at B and C, the prongs. The independence of the outlying nodules is now evident, as is also the fact that the growths connected with the mass, and raising it, have extended from beneath it, not from its edge. $\times \frac{3}{4}$.

PLATE 28

FIG. 7. Cross-section of the tip of the prong B of Fig. 6. The dark spot consists of the epithelial cells that have advanced furthest, but most of the tip consists of reactive connective tissue. It lies between an artery and a large vein (arrows). $\times 25$.

FIG. 8. Subcutaneous extensions from virus-induced papillomas in a cottontail. At A is the scar left by excision of a nodule situated in the position of the superficial inguinal gland. It was connected with the large subcutaneous mass by a narrow fibrous cord (see Fig. 13). The "pendule" at B was later excised (see Fig. 11). Owing to the way in which the animal was held, the skin is raised into a fold between the nodule C and the main mass, but actually no deep connection existed between them. $\times 1$.

FIG. 9. A completely keratinized papillomatous nodule in the lung of a domestic rabbit. For history see text. $\times 15$.

PLATE 29

FIG. 10. Sagittal section of the prong C of Fig. 6 and the tissue about it. It consists mostly of keratinized material, but is irregular in outline owing to the invasive activity of the living epithelium. Near its tip small cysts have formed secondarily, and just beyond these a large vein can be seen in oblique section (V). $\times 7\frac{1}{2}$.

FIG. 11. Section of the subcutaneous "pendule" of Fig. 8. It has been turned so that it appears to extend in a direction opposite to the real one. The growth consists mostly of keratinized epithelium and is characteristically papillomatous. Its papillae are introverted. $\times 4$.

FIG. 12. Papilloma growing in the inflamed pulmonary tissue of a domestic rabbit. For history see text. $\times 24$.

FIG. 13. Part of the fibrous cord connecting the large subcutaneous mass of Fig. 8 with the nodule found in the situation of the superficial inguinal gland. A profuse reactive tissue surrounds dead epithelium. $\times 165$.

FIG. 14. Subcutaneous extensions from an anaplastic squamous cell carcinoma arising in a papilloma, as seen from the under side. The growth followed the large vessels, which have lost their blood content, however, in consequence of the excision, and hence are not visible. For comparison with Figs. 3 to 6. $\times 1$.

FIG. 15. Longitudinal section of one of the finger-like extensions seen in Fig.

14. The growth was a squamous cell carcinoma with pronounced cystic tendencies. For comparison with Figs. 10 and 11. $\times 14$.

PLATE 30

FIGS. 16, 17, 18, and 19. The fate of emboli of papillomatous tissue in the domestic rabbit. The animal furnishing the sections was killed 5 days after an intravenous injection of bits of a growth induced on its skin by virus inoculation. In Fig. 16 invasion of the lung is actively under way, but the position of the original embolus can be told by the fragments of hair included with it. Fig. 17 shows a dying embolus and the vessel in which it has lodged. The epithelial cells have formed a syncytium. In Fig. 18 the embolus at the fork of an artery, has proliferated and rounded out, but has not yet become attached to the vessel wall. In Fig. 19 this has happened and invasion has begun. A nearby embolus has undergone cystic degeneration and is thickly encapsulated in connective tissue. $\times 91$.

PLATE 31

FIG. 20. A "papilloma of the second order," deriving from an ordinary papilloma induced with virus in a domestic rabbit. (All the later figures are from such animals). $\times 15$.

FIG. 21. A deep papilloma of uncertain status. The animal died on the 191st day after the inoculation of virus into a scarified square of skin. It had then a confluent papillomatous mass several centimeters across, on the skin surface, and underlying it and extending somewhat beyond its border a larger subcutaneous nodule like a flattened sphere. The figure is taken from a section through the edge of both growths. At many points they were directly joined, but in the region shown they are separated by the thin layer of the superficial corium (arrow), marked as such by numerous sebaceous glands and more or less cystic hair follicles. They have precisely the same morphology, which is that of a somewhat irregular, virus-induced papilloma. Intact epidermis overlies the deep growth. Hematoxylin and eosin. $\times 15$.

FIG. 22. Metastasis in a regional lymph gland from a malignant, cystic papilloma such as has been pictured in a previous paper (6). $\times 32\frac{1}{2}$.

FIG. 23. Implantation nodule on the parietal peritoneum, resulting from the intraperitoneal injection of a fragment of an ordinary papilloma procured 23 days after virus inoculation, shortly after the growth appeared. The animal was killed 26 days later. The growth had entered a blood vessel (*vide* Fig. 15 of reference 1). For comparison with Fig. 22. $\times 32\frac{1}{2}$.

PLATE 32

FIG. 24. Part of a metastasis of a cystic papilloma in a lymph node. $\times 15$.

FIG. 25. Another such metastasis. The resemblance to an experimental implant of an ordinary virus-induced papilloma is complete. $\times 15$.

FIG. 26. Still another such metastasis in cross-section, showing a living, introverted, papillomatous process and keratinized material deriving from other, similar processes. $\times 35$.

FIG. 27. The region X, of Fig. 10, as shown in an adjacent section. Save for the secondary cystic differentiation the growth resembles a squamous cell carcinoma. Much new-formed connective tissue surrounds it nearly everywhere, but at a few points it has advanced beyond this and lies amidst the voluntary muscle fibres (arrows). $\times 55$.

PLATE 33

FIGS. 28 and 29. Parts of the growths of Figs. 22 and 23 respectively under high magnification. The cell arrangement is somewhat more disorderly in each case than in the usual virus-induced papilloma, and the entire layer of living cells is somewhat flattened (1), but the differentiation is like that occurring in such growths. $\times 310$ and 500 respectively.

FIG. 30. A malignant, cystic papilloma that originated in papillomas due to tattooing virus into the skin low down on the side of a rabbit. Many ordinary papillomas of the same inoculation are present. All are deeply pigmented and some have coalesced. The malignant growth has extended under its neighbors and into the skin between them, forming cysts there, not a few of which are sub-epidermal. Secondary ulceration has taken place, and, owing to scirrhus shrinkage of the connective tissue the papillomas on the other side of the animal have been pulled over the ridge of the back-bone and hence are visible in the photograph. $\times 1$.

FIG. 31. The same malignant cystic papilloma at an earlier date. It has advanced almost to the axilla. $\times \frac{1}{2}$.



Photographed by Joseph B. Haulenbeeck

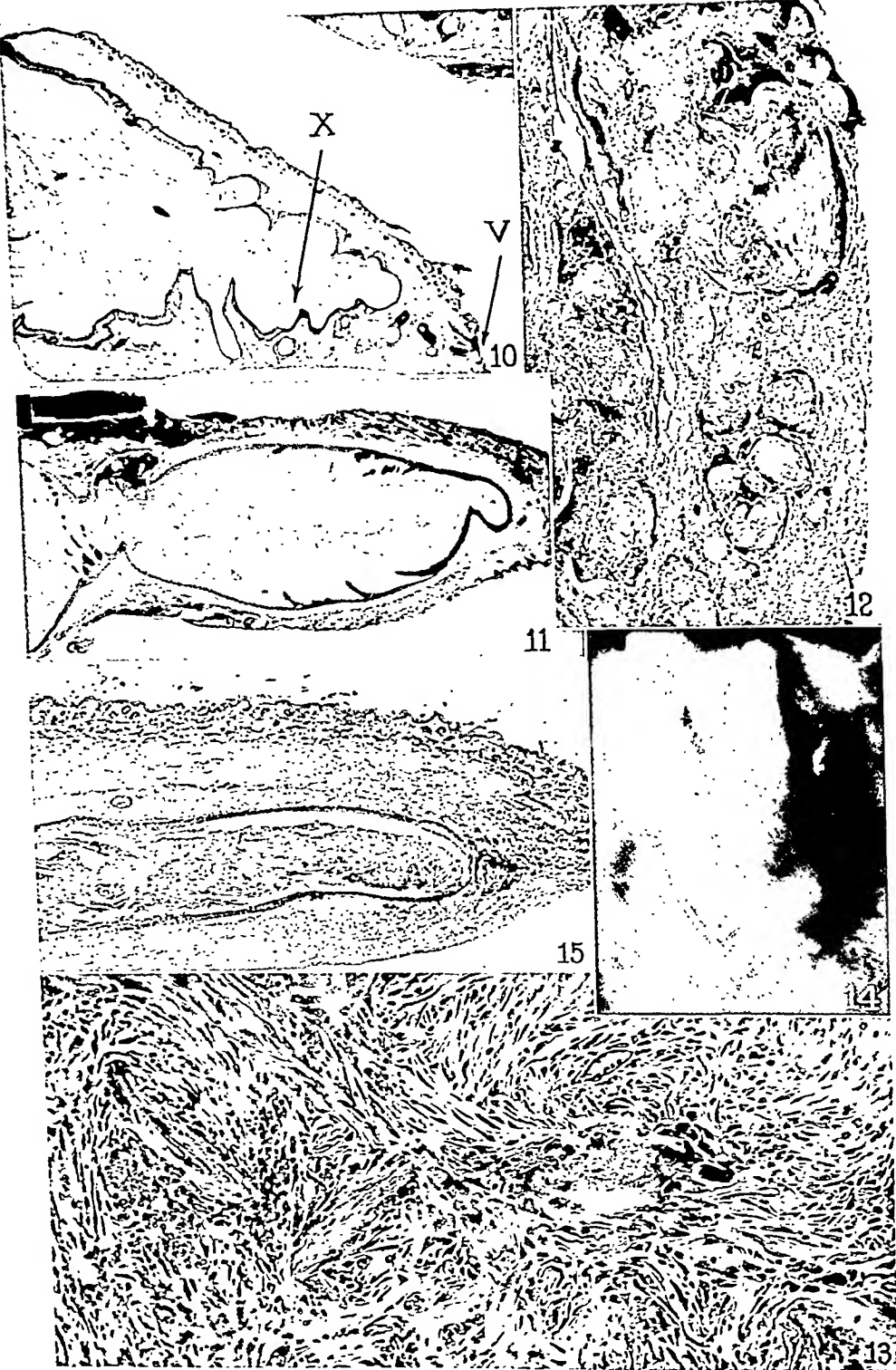
(Rous *et al.*: Cancers derived from papilloma virus. II)



Photographed by Louis Schmidt and Joseph B. Haulenbeck

(Rous *et al.*: Cancers derived from papilloma virus. II)





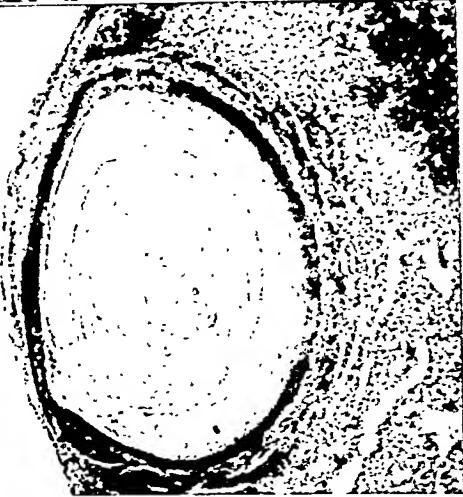
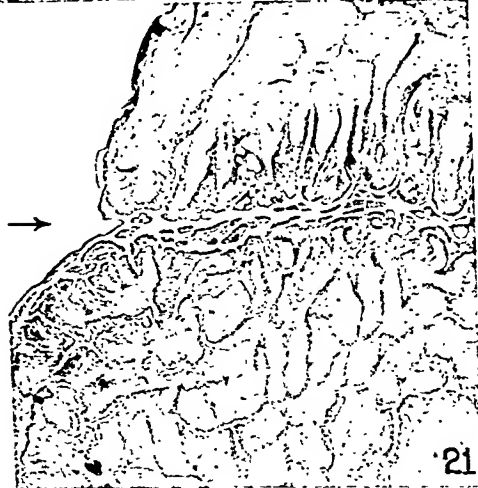
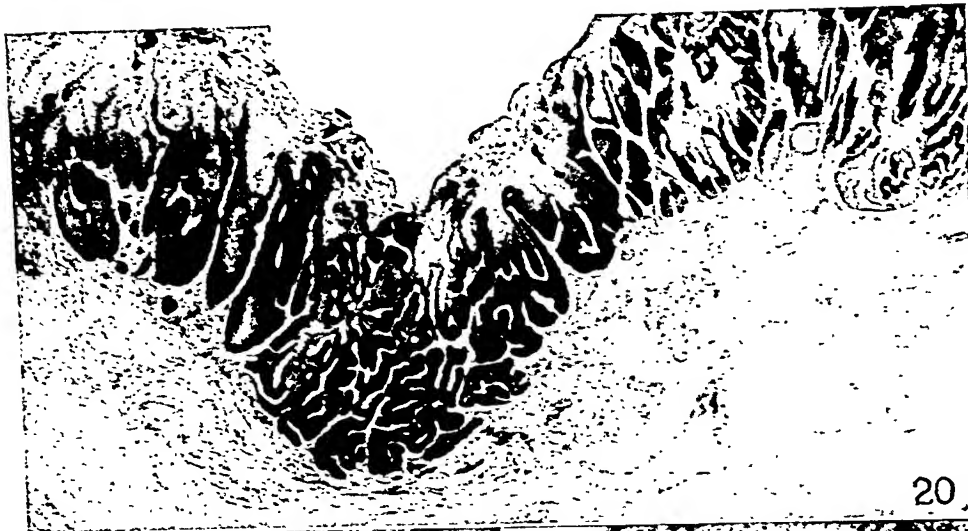
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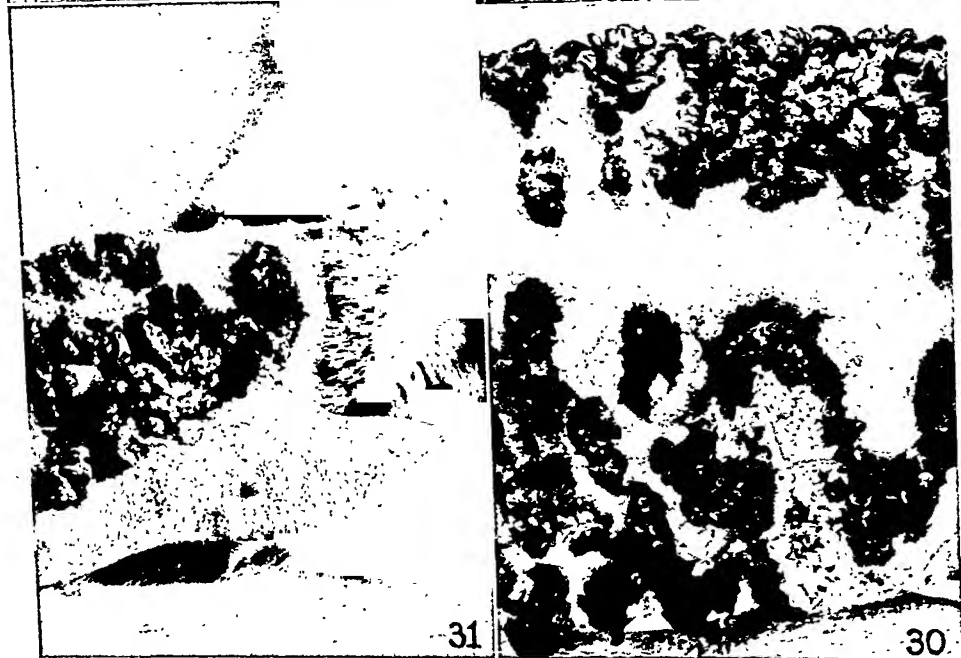
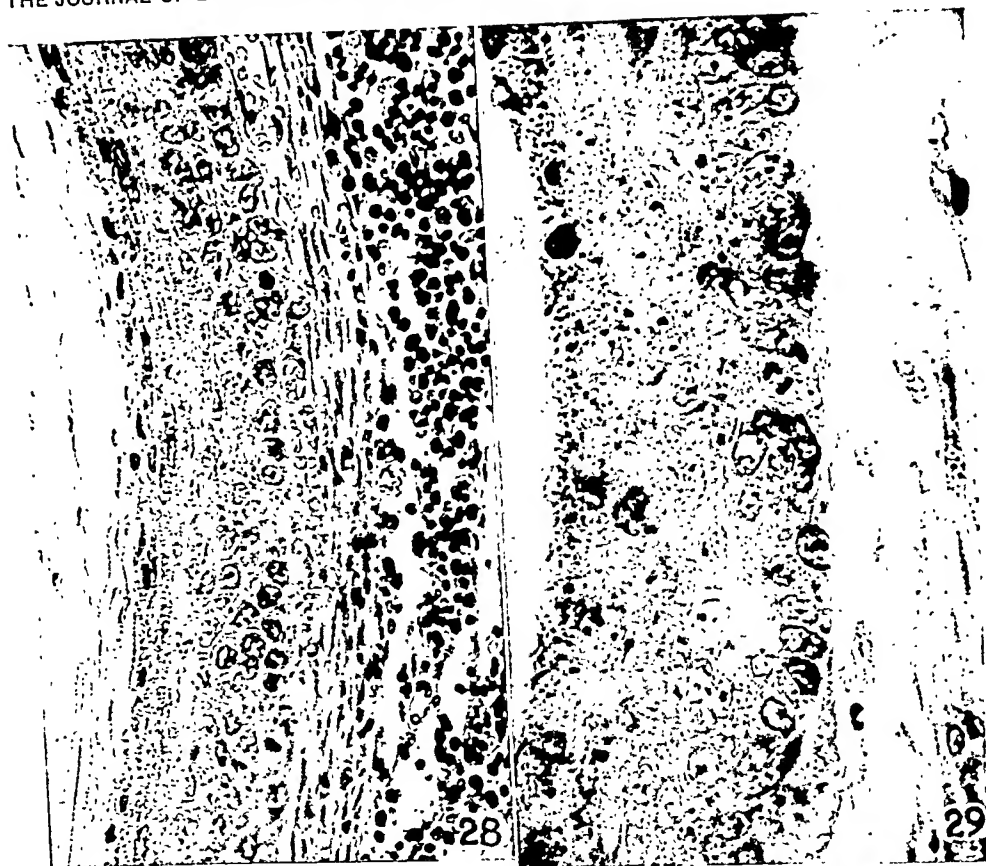


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(Rous *et al.*: Cancers derived from papilloma virus. II)

STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

IV. OBSERVATIONS ON A NON-TYPE SPECIFIC HUMORAL FACTOR INVOLVED IN RESISTANCE TO PNEUMOCOCCUS TYPE III

BY JOHN F. ENDERS, Ph.D., CHAO-JEN WU,* M.D., AND
MORRIS F. SHAFFER,* Ph.D.

*(From the Department of Bacteriology and Immunology, The Harvard
Medical School, Boston)*

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Experimental evidence presented in Paper III¹ indicates that removal and destruction of both rabbit virulent and avirulent strains of *Pneumococcus* Type III in the normal animal are brought about by the same means, namely, the phagocytic cells of the body, provided the integrity of the capsule becomes impaired. It leaves unanswered, however, the interesting question as to whether this phagocytosis is "spontaneous," that is, taking place without the intervention of a serum component, or whether the adjuvant action of an antibody is essential to the process. The following experiments although they fail to give a complete answer do nevertheless reveal the participation of an antibody reacting with the somatic C carbohydrate of Tillett, Goebel and Avery (1) which not only appears to play a rôle in the phagocytosis of normal animals but is probably responsible for bringing about the non-type specific immunity which Tillett (2) obtained in rabbits vaccinated with R pneumococcus or the smooth form of a heterologous type.

Methods and Materials

The strains of *Pneumococcus* Type III and the R variants derived from them were the same as those mentioned in Paper I.²

* At the time this work was done, Dr. Wu held a Travelling Fellowship from the Peiping Union Medical School, and Dr. Shaffer a Fellowship in the Medical Sciences from the National Research Council.

¹ Enders, J. F., Shaffer, M. F., and Wu, C.-J., *J. Exp. Med.*, 1936, 64, 306.

² Enders, J. F., and Shaffer, M. F., *J. Exp. Med.*, 1936, 64, 7.

The C carbohydrate of the pneumococcus was prepared according to the procedure of Tillett, Goebel and Avery from an R variant obtained from our stock strain of *Pneumococcus* Type I.

The soluble specific substance was prepared from cultures of strain CH according to the procedure of Heidelberger, Goebel and Avery (3).

Anti-R pneumococcus serum was produced in rabbits according to the method described in Paper I.

References to the techniques used in the phagocytic work have been noted in Papers II³ and III.

Effect of C Carbohydrate on the Phagocytosis of Pneumococcus Type III by Normal Cells and Serum

The antiphagocytic effect of the type specific carbohydrate in systems containing the homologous antibody has been demonstrated by such studies as those of Sia (4) and Ward and Enders (5). This antibody, however, appears to be entirely absent in the normal rabbit as indicated by the work of Tillett (6), and the observations recorded below concerning the effect of the type specific carbohydrate on the phagocytosis of Type III pneumococcus in the rabbit system. We were induced, therefore, to make a further study of the antibody reacting with another known antigenic constituent of this organism, namely, the C carbohydrate or species specific polysaccharide of Tillett, Goebel and Avery. Particular attention was directed to this fraction and its antibody, since the failure of Tillett to produce any evidence of increased resistance after injection of pneumococcus nucleoprotein would seem to eliminate the homologous antibody from any participation in the natural immunity of the rabbit or that which ensues when intact R forms or heterologous S strains are employed as vaccines.

In Table I, the effect of adding 1 drop of the C carbohydrate on the phagocytosis of old cultures of strain SV in 0.25 cc. of a suspension of the leucocytes and serum of a normal rabbit is shown. The number of organisms from a 22 hour culture taken up by the cells in the absence of C is reduced about 33 per cent when this substance in a dilution of 1:200 is added. A concentration of 1:1,000 exhibits no depressive action on them, but does influence the ingestion of the 30 hour cultures. In another experiment, when a culture much younger than these (10 hour SV and CH) was used, the effect of the addition of the C carbohydrate was

³ Shaffer, M. F., Enders, J. F., and Wu, C.-J., *J. Exp. Med.*, 1936, 64, 281.

even more pronounced. The introduction of the type specific carbohydrate does not reduce the phagocytosis compared with the control of normal serum only. In the lower half of the table are included figures obtained from an experiment in which the C carbohydrate was added to normal human defibrinated blood. Here, its effect is very pronounced when the strain used is SV. With CH, its action though definite is less marked. In other experiments of the same kind, smaller

TABLE I

Effect of C Carbohydrate on the Phagocytosis of Pneumococcus Type III by the Normal Serum and Polymorphonuclear Leucocytes of Man and the Rabbit

Strain	Age of culture	Material tested	Average No. of cocci per 10 cells	Cells containing cocci	No. cells counted
	hrs.			per cent	
SV	22	—	11.2	29.0	300
SV	22	C 1:200	7.5	24.2	264
SV	22	C 1:1,000	13.3	40.0	200
SV	22	SSS III 1:1,000	12.3	33.0	200
SV	30	—	15.4	43.0	210
SV	30	C 1:200	10.0	33.0	200
SV	30	C 1:1,000	10.8	27.0	150
SV	30	SSS III 1:1,000	14.9	40.0	200
*SV	10	—	18.4	32.4	250
SV	10	C 1:200	5.4	11.6	500
*CH	10	—	37.1	50.8	250
CH	10	C 1:200	17.1	32.4	250
SV	18	—	61.0	94.0	50
SV	18	C 1:100	0.2	2.0	50
SV	18	SSS III 1:100	0.0	0.0	50
CH	18	—	221.6	100.0	50
CH	18	C 1:100	141.2	94.0	50
CH	18	SSS III 1:100	32.2	58.0	50

Normal rabbit
cells and
serum

Normal human
cells and
serum

* The data on the 10 hour cultures of CH and SV were obtained in another experiment, employing the cells and serum of a different rabbit.

quantities of the material have been employed. These depress the phagocytosis of strain SV of this age in dilutions as high as 1:10,000 to 1:100,000. Little or no reduction in the numbers of strain CH ingested was brought about by these quantities. It is evident from the data that the type specific carbohydrate also has a similar action, reducing the numbers of strain SV ingested to zero and those of strain CH about seven times. To us this appears to denote that in the particular human blood used, both type specific and anti-C antibody were present.

To opsonize this 18 hour culture of SV with its greater quantity of capsular material, the combined concentrations of both antibodies were necessary. The available amount of either one was by itself insufficient to bring this about. Since in the CH organism of this age the loss of capsular constituents is relatively much greater, opsonization may be accomplished by the concentration of the single antibody which remains after the elimination of the other by addition of its homologous antigen. Indeed, from information derived from unrecorded experiments it is probable that certain CH cocci have become at this stage of cultivation so susceptible to the attack of phagocytes that they can be ingested in the absence of sensitization by either the anti-SSS III or anti-C, or anti-P antibodies, since the addition of all these fractions together fails to remove entirely the phagocytic capacity of the system. Such organisms behave like the R variant, for we have found that the addition of these three known antigens of the pneumococcus has practically no effect on the phagocytosis of R forms by the cells and serum of normal rabbits. These facts we consider may also serve to explain the residual phagocytosis seen after addition of the C carbohydrate to the rabbit system containing 22 hour cultures of strain SV. It is entirely possible that the cocci found within cells under these conditions represent forms so far degraded in respect to the antigenic properties of the surface that the anti-C antibody is not necessary for their opsonization. To eliminate the possibility that the action of the C carbohydrate consisted in fixation of complement, rather than in union with and neutralization of an opsonin of the serum, the titre of hemolytic complement in the system was determined. No diminution of complement ensued upon the addition of the C substance in the quantities employed.

On the basis of these results, it can be stated that an antibody or antibody-like principle in the normal sera of rabbit and man reacting with the C carbohydrate of the pneumococcus has a share in the opsonization of both rabbit virulent and avirulent smooth strains of *Pneumococcus* III, provided the capsule has become impaired, for it will be recalled from the data presented in Papers II and III that when young and fully capsulated, both these organisms are completely resistant to phagocytosis in human and rabbit systems.

The Opsonic Property of Anti-R Pneumococcus Serum

The fact that an antibody reacting with the C carbohydrate in normal serum contributed to the opsonization of the pneumococcus, together with the demonstration by Tillett of active and passive immunity in rabbits following injection of R vaccine or anti-R blood or serum, led us to determine whether or not a serum prepared in rabbits

following prolonged treatment with heat-killed R pneumococci could increase the phagocytosis of strains CH and SV.

The usual technique was modified in the instances where the R serum was tested by mixing equal volumes of this serum and fresh normal rabbit serum. This mixture was added to the exudative leucocytes and the red cells which had been previously removed from the normal serum. In this way the antiserum

TABLE II

The Opsonic Effect of Anti-R Pneumococcus Serum on the Phagocytosis of Pneumococcus Type III by Rabbit Leucocytes

Strain	Age of culture	Constituents of the system	Average No. of cocci per 10 cells	Cells containing cocci	No. cells counted
	<i>hrs.</i>			<i>per cent</i>	
CH	6	Normal serum	5.1	9.0	200
CH	6	Normal serum + anti-R serum	10.2	17.0	200
CH	6	Normal serum + anti-R serum + C 1:1,000	1.7	3.0	200
CH	16	Normal serum	87.0	86.0	50
CH	16	Normal serum + anti-R serum	128.0	96.0	50
CH	16	Normal serum + anti-R serum + C 1:1,000	69.3	79.0	100
SV	16	Normal serum	7.6	18.5	400
SV	16	Normal serum + anti-R serum	15.2	34.0	400
SV	16	Normal serum + anti-R serum + C 1:1,000	5.1	15.0	400
CH-R	6	Normal serum	385.0	100.0	10
CH-R	6	Normal serum + anti-R serum	338.0	100.0	10
CH-R	6	Normal serum + anti-R serum + C 1:1,000	250.0	100.0	10
SV-R	6	Normal serum	108.8	84.0	25
SV-R	6	Normal serum + anti-R serum	158.8	96.0	25
SV-R	6	Normal serum + anti-R serum + C 1:1,000	140.0	97.0	30

could be obtained in sufficient concentration to be effective. From the data assembled in Table II, it will be seen that in the presence of anti-R serum the phagocytosis of a 6 hour culture of CH is increased 100 per cent, that of a 16 hour culture about 47 per cent. The number of 16 hour SV organisms ingested is also increased by 100 per cent. Addition of 1 drop of 1:1,000 dilution of the C carbohydrate to the mixtures containing the R serum reduces the number of organisms phagocytosed to a figure which is less than that of the normal serum. The reduction

is particularly marked in the case of the 6 hour CH culture. Here, as with strain SV in the normal human serum, the greater effect of this substance on the younger cultures may, in part, depend upon the amount of surface antigen in relation to the quantity of available antibody. In the older culture, antigen is reduced and the small amount of antibody which may remain unbound after introduction of the C carbohydrate may be still sufficient for sensitization of a considerable number of organisms. Moreover, in older cultures, completely degraded forms not requiring the C antibody for opsonization have doubtless developed. Had greater concentrations of C carbohydrate been employed in this experiment, the results might have been even more striking.

To obtain information concerning the influence of the anti-R serum on R forms of these strains the same type of experiment was done using 6 hour cultures of the R variants derived from each. It is evident that the serum has no effect on the phagocytosis of that obtained from strain CH. There is possibly some enhancement of the phagocytosis of strain SV-R, but because of the extremely large number of intracellular organisms present in all cases, and the small number of cells counted, no conclusive evidence is afforded of any definite action on the R organisms of this antibody. It is certain that extensive ingestion of these forms may occur in its absence.

Taken as a whole, these experiments indicate that the anti-C antibody present in the serum of rabbits immunized with R vaccine can increase the phagocytosis of the rabbit virulent and avirulent pneumococci, provided these be obtained from cultures in which the capsules of the organisms are no longer intact.

The Protective Action in Mice of Anti-R Pneumococcus Rabbit Serum

The previous experiments, since they revealed a certain amount of opsonic activity attributable to the anti-C carbohydrate antibody, induced us to ascertain whether or not this entity could in the animal body act in the capacity of a protective agent.

To this end a series of mice were injected intraperitoneally with 0.5 cc. of anti-R pneumococcus rabbit serum prepared by repeated injections of heat-killed R pneumococci, derived from a strain of *Pneumococcus* Type I, as noted in the section on technique. A second series of animals was given the same quantity of the same anti-R serum, from which the precipitin reacting with the C carbohydrate had been removed by repeated precipitations at the point at which maximum flocculation occurred. The original serum gave a positive ring test with a dilution of 1:10,000 of the carbohydrate. After absorption of precipitin, no reaction was obtained using this technique with dilutions of the carbohydrate ranging from 1:100 to 1:100,000. A third series of animals was injected with 0.5 cc. of the

anti-R serum, which had been previously treated with fresh guinea pig kidney with the object of removing the Forssman antibody which has been shown by Powell and coworkers (7), to increase in rabbits the therapeutic action of type specific pneumococcus antibody. Before absorption, the anti-R pneumococcus serum hemolyzed sheep cells in a dilution of 1:800. After absorption it failed

TABLE III

The Protective Action in Mice of Anti-R Pneumococcus Sera against Pneumococcus Type III and the Effect of Absorbing Such Sera with C Carbohydrate and Guinea Pig Kidney

	Group 1 0.5 cc. anti-R rabbit serum intraperi- toneally		Group 2 0.5 cc. anti-R rabbit serum absorbed with C intraperi- toneally		Group 3 0.5 cc. anti-R rabbit serum absorbed with guinea pig kidney intraperi- toneally		Group 4 0.5 cc. normal rabbit serum intraperi- toneally		Group 5 Untreated		Remarks
	S	D	S	D	S	D	S	D	S	D	
Experiment 1	9	1	2	8	nd	nd	1	9	nd	nd	Groups 1, 2, 4 received approx- imately 5,800 Pn intravenously Groups 1, 2, 3, 4 received approxi- mately 3,400 Pn intravenously. Group 5 received approximately 34 Pn intravenously
Experiment 2	5	5	0	11	7	3	4	7	1	3	
Totals.....	14	6	2	19	7	3	5	16	1	3	

All mice injected intravenously with 0.2 cc. of dilution of 6 hour broth culture of strain SV.

In the case of each mouse that died a culture of the heart's blood revealed the presence of pneumococcus.

to do so in a dilution of 1:5,—the lowest dilution tested. 0.5 cc. of normal rabbit serum was injected into a fourth series of mice. 24 hours later all the mice were injected intravenously with 0.2 cc. of a 10^{-4} dilution of a 6 hour broth culture of strain SV. Intravenous inoculation was employed since it was found that little or no protection was demonstrable when the culture was injected into the peritoneum. The results of two experiments, in only one of which, however, was the serum absorbed with guinea pig kidney employed, are summarized in Table III. Al-

information concerning the mechanism by which the rabbit virulent organisms may be resisted in the absence of the type specific antibody. We lack, however, complete data concerning the humoral factors which may participate in the removal by the normal animal of either this strain or the less virulent strain CH, although the depression of phagocytosis in normal cells and serum by the C carbohydrate points to a participation of the corresponding antibody. Therefore, the attempt was made to reduce the resistance of the normal mouse to strain CH inoculated intravenously by an antecedent injection of the C carbohydrate with a view to reducing or removing in the animal any natural antibody which might react with it, since a similar procedure had been found effective in removing the immunity induced by previous vaccination with *Pneumococcus* Type I acetyl polysaccharide (Enders and Wu (8)).

Twenty mice were injected intraperitoneally with 0.5 cc. of 1:500 dilution of the C carbohydrate in physiological saline. Another lot was injected with the same quantity of saline as controls. 3 hours afterwards, the group receiving C carbohydrate was divided into four lots of five mice each. Each lot was injected with 0.2 cc. of broth dilutions of 6 hour cultures of strain CH, falling from 1:100 to 1:100,000 by tenfold quantities. The controls were divided into lots of four each and injected with dilutions from 1:10 to 1:100,000 of the same culture. No significant differences were found in the number of deaths that occurred in lots receiving the same quantity of organisms, but one of which had been previously treated with the C carbohydrate.

This failure to demonstrate any depression of the resistance of the normal mouse to strain CH by preliminary injection of the C substance may be attributed either to the nonparticipation of the corresponding antibody in this protection, or to its incomplete removal by the amount of C carbohydrate administered. We are inclined to regard the last hypothesis as more plausible.

DISCUSSION

Although the C carbohydrate of the pneumococcus was isolated 6 years ago, little study has been devoted to its possible rôle in the mechanisms of immunity. It is known that this material forms a precipitate with its homologous antibody *in vitro*, and that its distribution among bacteria is apparently not limited to the pneumo-

coccus alone, since cross reactions have been obtained, for example, with antimeningococcus serum. Furthermore, observations by Tillett and Francis (9), and by Finland and Dowling (10) show that when the C substance is injected intradermally into patients in the early stages of pneumonia an inflammatory reaction occurs. Subsequent to the time of crisis, the material fails to elicit this response. These facts suggest that an antibody reacting with the C substance is present during the period of acute illness, but reveal nothing as to its function.

Before the discovery of the C carbohydrate, it was demonstrated that resistance in rabbits against virulent Type III pneumococci developed following the injection of vaccines consisting of R organisms, or those of heterologous smooth types. Such resistance could be passively transferred to other rabbits with the blood or serum of those actively immunized. Tillett (2) believed that this protection did not depend upon the presence of the antipneumococcus protein antibody, or indeed upon the presence of any antibody, since his experiments showed that, whereas pure nucleoprotein (at that time the only species antigen recognized), or solutions of pneumococci, produced high titres of this antibody, but exhibited no protective power, the injection of intact R pneumococci or the smooth organisms of a heterologous type resulted in the appearance of protective properties in the serum.

The experiments described in this paper not only serve to suggest that the C carbohydrate and its corresponding antibody are involved in this non-type specific immunity against the pneumococcus, but, also, in fulfillment of their immediate objective afford an understanding of at least one of the humoral factors concerned in the defense of the normal animal against the two strains of *Pneumococcus* Type III, which are our particular concern. Specifically, they have shown that the C carbohydrate obtained from cultures of a rough variant of *Pneumococcus* Type I may inhibit the opsonic properties of the normal serum of man and the rabbit, which promote the phagocytosis of virulent Type III pneumococcus. Furthermore, it has been found that an antiserum prepared against the same R variant of *Pneumococcus* Type I has a definite, though limited, opsonizing effect upon the smooth Type III organisms, which is abolished in the presence of a suitable quantity of the C carbohydrate. It is to be emphasized that the anti-C antibody whether present in normal or immune serum

is effective *in vitro* as an opsonin only against organisms possessing imperfect capsules. Evidence that the antibody is active, not merely in the test tube, but also in the animal body is presented by experiments in which the anti-R serum injected into one lot of mice apparently affords protection against a subsequent dose of the rabbit virulent *Pneumococcus* Type III provided these be inoculated by the intravenous route, while introduction of the same antiserum absorbed with the C carbohydrate fails to induce any increase in the resistance of the animals. The results of attempts to actively immunize mice against this strain by repeated injection of R *pneumococcus* vaccine have proved inconclusive, but suggest that a slight depression of susceptibility to infection occurs in some animals. Injection of the C substance into normal mice previous to intravenous infection with the rabbit avirulent strain failed to reduce the high resistance of the animals. It also proved impossible by preliminary administration of the anti-R serum to increase the resistance of normal mice against CH.

In spite of these failures, we believe that taken as a whole, the findings indicate that when the capsule of either strain has become slightly damaged, the anti-C antibody, which is present in normal animals in small amounts, may exert an opsonizing action which results in phagocytosis of the organisms, either by the leucocytes or the fixed tissue cells, as has been shown to occur (*cf.* Paper III). Since alterations in the capsule of the rabbit avirulent strain have been demonstrated to take place more rapidly, and in a given time are more extensive, it is most probable that smaller quantities of this antibody are more effective against this strain than against the rabbit virulent strain, which maintains a large capsule for many hours. It is, however, also clear that *in vitro* capsular degradation may proceed to such an extent that sensitization with the anti-C antibody is not necessary as a preliminary step in the process of phagocytosis. During the natural course of infection this state is probably seldom attained, since before it is reached, opsonization by the anti-C antibody would have taken place with the resulting elimination of the organism.

If these findings prove to be correct and are found to have a more extensive application, one might possibly regard the anti-C carbohydrate antibody as an important factor in the natural immunity of individuals to infection not only with small doses of pneumococci,

but with other organisms such as the meningococcus which possess, if not an identical, at least a similar antigenic constituent. Indeed, it does not appear illogical to think that this antibody may function successfully in protecting from infection those persons such as practically all infants, many young children and some adults, who have in their serum no naturally occurring antibody corresponding to the type specific antigen.

CONCLUSIONS

1. The phagocytosis of rabbit virulent and rabbit avirulent strains of *Pneumococcus* Type III by the cells and serum of both man and the normal rabbit is decreased by the previous addition of the C carbohydrate of Tillett, Goebel and Avery.

2. The addition of anti-R pneumococcus serum to normal rabbit serum and cells results in increased phagocytosis of these strains, which is removed by the C carbohydrate.

3. Anti-R pneumococcus rabbit serum will protect mice against 100 or more M.L.D. of the rabbit virulent strain of *Pneumococcus* Type III, provided the organisms are injected by the intravenous route.

4. Absorption of anti-R pneumococcus rabbit serum with the C carbohydrate removes the mouse protective property.

5. The application of these findings to the mechanism responsible for the removal of the rabbit virulent and avirulent strains from the circulating blood of the normal animal is discussed.

SUMMARY AND GENERAL CONCLUSIONS

Since there is no evidence for the occurrence of type specific antibody in the normal rabbit and since, as we have shown, the *Pneumococcus* Type III whether avirulent or virulent is not removed from the blood stream or destroyed when the capsule is intact, the following factors which have been revealed in the course of our work appear to represent certain essential components, if not the complete mechanism, upon which the natural immunity of the rabbit against this organism depends. (a) The elevation of the body temperature after intravenous infection to 41°C. or thereabouts and its maintenance for varying periods. (b) The ability of the phagocytic cells, both fixed

and mobile, to attack any cocci which have become vulnerable through the deterioration of capsular integrity. (c) The adjuvant effect of an antibody, reacting with the somatic C carbohydrate, which enhances the phagocytosis of such organisms as no longer possess a completely intact envelope.

Conversely, the varying degrees of virulence for rabbits observed among *Pneumococcus* Type III strains are based upon: (a) differences in the ability of the organisms to multiply at the elevated temperatures encountered in the infected host. Strains markedly susceptible to the harmful influence of this factor fail to induce a generalized fatal infection. Not all "thermo-resistant" strains are highly virulent, however, and these may contrast sharply with regard to (b) size of the capsule and the ease with which it is impaired or completely lost. The capsules must be maintained intact for a sufficient time until multiplication of the organisms can proceed to such a degree that death of the host results. Avirulent strains even when capable of growth at 41°C. appear to be unable to satisfy this requirement.

The differences in virulence of various strains apparently conditioned by these factors are not limited solely to the case of the rabbit, since for at least two strains similar differences in virulence have been shown to exist when the intravenous route of infection is employed in mice.

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IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

IV. STATISTICAL STUDIES OF ELEMENTARY BODIES IN RELATION TO INFECTION AND AGGLUTINATION*

BY ROBERT F. PARKER, M.D., AND THOMAS M. RIVERS, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The exact relation of the elementary bodies of vaccinia to the causative agent of the disease has long been a subject of discussion. Recent observations of others as well as those made by us indicate that it is an intimate one, and that in general the infectivity of a suspension is related to the number of elementary bodies contained therein (1, 2, 3). Furthermore, the agglutinating properties of a suspension are related to the number of elementary bodies present. However, exact observations are lacking, and since, because of technical difficulties, the possibility of obtaining direct proof of infectivity by the inoculation of a single elementary body seems remote, the present work was undertaken with the object of applying statistical methods to the elucidation of the problems.

Methods and Materials

Preparation of Elementary Body Suspensions.—The details concerning the manner of preparation of pure suspensions of elementary bodies have already been given (3). For our present work suspensions differing as widely as possible in content of elementary bodies were desired, and these were obtained in the following manner. Suspensions of elementary bodies were prepared in the usual way, and subjected to sedimentation in an angle centrifuge for different periods of time. The various supernatant fluids, containing different amounts of elementary bodies were decanted and saved, while the sediments were resuspended in reduced vol-

* The authors wish to express their thanks to Dr. Hugo Muench for his valuable assistance in the application of statistical methods.

umes of fluid. In such preparations there was a 10,000-fold variation in the number of elementary bodies. The purity of all suspensions was confirmed by examination of smears stained by the Morosow technic.

Counting of Elementary Bodies.—The elementary bodies were counted in a Petroff-Hausser counting chamber, fitted with a reinforced cover slip. By means of dark field illumination and proper combination of lenses (20 × ocular, 20 × 8.3 mm. objective), the elementary bodies were readily identified. It was necessary to cleanse the chamber carefully between each count, and remove all traces of oil from the surfaces. Doubly distilled water was used for making all dilutions of suspensions of elementary bodies to be counted. It is to be noted that distilled water and physiological solution of sodium chloride are suitable for making dilutions of suspensions of elementary bodies provided such diluted suspensions are not centrifuged; if they are centrifuged, the elementary bodies can be re-suspended only with difficulty, if at all, because an irreversible agglutination is found to have taken place. Furthermore, the agglutination becomes more pronounced with each repetition of the washing of the elementary bodies.

Titration of Virus.—Titrations were made by means of intradermal inoculation in rabbits of 0.25 cc. of serial dilutions of virus. In order to control the effect of variation exhibited by different rabbits, as well as variation due to the use of different sites of inoculation in the same rabbit, the titrations were carried out in the following manner. Four samples of virus were usually titrated at the same time on four rabbits; each sample was titrated once on each rabbit and the sites of inoculation were varied so that each sample appeared once in each of the four possible sites of inoculation, *viz.*, upper left side, lower left side, etc. Because of the regular appearance of generalized lesions caused by the strain of virus used, observations were terminated on the 5th day.

Gates Densitometer.—The densitometer used was of the type described by Gates (4), which is a simple instrument for measuring the depth to which a loop of 18 gauge nichrome wire must be submerged in a turbid fluid to cause it to become invisible when viewed from above. Our observations were made in subdued daylight with a black surface below the tube (internal diameter 14 mm.) containing the suspension.

Agglutination Reaction.—The manner of performing the agglutination reaction has already been described (3). In these experiments serial dilutions of suspensions of elementary bodies were mixed with varying dilutions of antiserum in order to determine the greatest dilution of the suspensions in which a visible agglutination still occurred. In a similar manner the dilution of elementary bodies was determined which was optimum for titration of serum.

EXPERIMENTAL

In the present investigation we have examined statistically a method of counting elementary bodies of vaccinia by direct observation and a rapid method for measuring the density of a suspension of them.

We have also studied the correlation between the elementary body content of a suspension and its infectivity. Finally, the number of elementary bodies required to produce a visible agglutination in the presence of immune serum was estimated, and the optimum number for titration of the agglutinating activity of serum was determined.

Direct Enumeration of Particles

It has been shown in counts of yeast cells (5) that the distribution of the organisms in the squares of the counting chamber is by chance,

TABLE I
Distribution of Particles in Counting Chamber

No. of particles per square	Squares containing given number of particles— observed	Squares containing given number of particles— expected	$\frac{x^2}{n}$
5 or less	8	11	0.82
6	8	9	0.11
7	15	12	0.75
8	17	13	1.20
9	15	13	0.31
10	10	12	0.33
11	8	10	0.40
12	10	7	1.30
13 or more	9	12	0.75
	100		$\chi^2 = 5.97$ $P = .59$
Mean.....	9.04		

and the number of squares containing a given number of organisms, the mean being known, may be predicted by means of Poisson's law of small chances. In order to determine whether such a distribution occurs also in the case of elementary bodies, which require dark field illumination to be made visible and are in constant active Brownian movement, the following experiment was performed.

Experiment 1.—A suspension of elementary bodies was prepared and diluted in a manner that would yield a concentration of elementary bodies suitable for counting. The chamber was charged with this suspension and the number of particles contained in each of 100 small squares (1/400 sq. mm.) was then determined. The experiment was repeated several times. The results of one count which is typical of all are set forth in Table I.

It will be seen from the results shown in Table I that the distribution observed agrees very well with that expected; the probability that the deviation observed arose from chance alone being 59/100.

The standard deviation (σ) of a Poisson series is $\sqrt{\text{mean}}$, in this case $\sqrt{9.04} = 3.0$, and, if the technic of counting is perfect, in a series of that size we should expect this to agree with the standard deviation as calculated from the squares of the deviations from the mean. We find, however, that the observed standard deviation is 2.76, slightly lower than the theoretical. This suggests that the chief source of error is due to excessive grouping of counts about a mid value, with a deficiency of low and high values. This discrepancy, however, is not sufficient to cast doubt on the value of the method, and one may reasonably assume that the distribution of particles follows Poisson's law, and that the technic of counting is satisfactory. It is then possible to estimate the accuracy to be expected. The

standard deviation of the mean of a Poisson series is $\sqrt{\frac{\text{mean}}{\text{number of counts}}}$; in this case $\sqrt{0.0902}$ or 0.30. If no source of error other than the variation due to sampling entered into the variance of the mean of counts based on the observation of 100 squares, we should expect the values for the various means to be distributed normally, and the standard deviation calculated from the mean squares to agree with the standard deviation expected in a Poisson series. The observed standard deviation of a series of 10 counts, each based on the observation of 100 squares, is however 0.85, while that expected is 0.305. We take, therefore, as the best estimate of the standard deviation of the mean $\sigma_m = \frac{0.85}{\sqrt{N}}$. For a set of 5 counts we have $\sigma_m = 0.380$.

In the process of cleansing the counting chamber it was found impossible entirely to remove particles which could not be distinguished from elementary bodies. Since the inclusion of such debris in the final estimate of elementary bodies would materially affect the results, and would moreover do so in an irregular manner, it seemed desirable to estimate the magnitude of the error caused by the debris. This was done in the following manner.

Experiment 2.—Three dilutions of an elementary body suspension were prepared of such density that the average number of particles per square was about 3, 6, and 9. In order to obtain such counts it was necessary to dilute the original suspension 400-, 200-, and 100-fold respectively. 10 counts of each dilution were made, the results of which are summarized in Table II.

From the results shown in Table II it will be seen that the difference between counts made at various dilutions is much more than can be

accounted for on the basis of random sampling. Such an error is most likely to be due to the inclusion in the counts of extraneous particles present in the diluent and in the counting chamber, but unrelated to the sample under observation. This possibility may be tested, and the magnitude of the error involved can be estimated by a simple procedure.

If in the least diluted sample P represents the number of elementary bodies present, and p the extraneous particles indicated above, then the observed count may be represented as $P + p$. With serial twofold dilutions we have counts of $\frac{1}{2}P + p$, $\frac{1}{4}P + p$, etc., i.e., p remains a constant in the original enumeration. After multiplying these counts by the dilution to determine the total number of particles present per square in the least diluted sample we have $P + p$, $P + 2p$, $P + 4p$, etc., from which the value of p may easily be found. Substituting the

TABLE II
Comparison of Counts Made at Different Dilutions of a Suspension

Dilution of original suspension	Mean No. of particles per square	Standard deviation	Calculated No. of particles per square of undiluted suspension	Corrected value, particles per square
100	9.32	0.97	932	774
200	5.64	0.75	1128	812
400	3.41	0.58	1364	732

observed values shown in Table II we find that for the original count, p = about 1.58. Application of this correction yields the figures of column 5 which agree more closely with each other.

We may conclude that a more accurate estimate of the number of elementary bodies in a suspension has been obtained when the figures are corrected for the inevitable presence of debris in the chamber.

Enumeration of Infectious Units

In the estimation of the concentration of infectious units in a given suspension of vaccine virus, the method usually employed is the following. Serial tenfold dilutions are prepared and a portion of each dilution is injected intradermally into a rabbit. After a suitable period, note is made of the highest dilution of the suspension which has given rise to a lesion, and the content of virus in the original suspension is said to be represented by the reciprocal of this dilution.

Thus if the highest dilution giving rise to a lesion is 10^{-6} , the suspension is said to contain 10^6 infectious units per unit volume inoculated. While suitable for ordinary estimations of the amount of virus, this method does not lend itself to exact determinations without some modification, because of the relatively large influence of chance variation.

If more than one titration is made, the influence of chance is obviously decreased, and the result correspondingly more reliable, the reliability being in general proportional to the square root of the number of observations which enter into the estimation. If the end-titer alone is recorded, and a logarithmic average calculated, the maximum number of observations included is obviously the same as the number of titrations performed, and a single widely variant result

TABLE III
Calculation of 50 Per Cent End-Point in Titration of Virus

Logarithm of dilution of virus	Inoculations positive	Inoculations negative	Accumulation—positive	Accumulation—negative	Per cent positive
3	4	0	12	0	100
4	4	0	8	0	100
5	3	1	4	1	80
6	1	3	1	4	20
7	0	4	0	8	0

may have a marked effect on the mean. It is possible, however, to increase to a large extent the number of observations utilized in a given number of titrations by choosing a different end-point, *viz.*, one at which 50 per cent of the inoculations may be expected to be positive, and 50 per cent negative (6). This point is probably the most stable one that can be selected and the one at which the chance variation of a single determination would have the least influence. The method of its calculation is illustrated in Table III, and the procedure is carried out as follows:

The first step is to separate for each dilution the inoculations causing positive from those causing negative results. Then the positive and negative results respectively are accumulated, the direction in which the accumulation is made being of primary importance. Each column is added, beginning with the smaller

end. The various sums in each column then represent the number of inoculations positive at that dilution and higher dilutions or the number negative at that dilution and lower dilutions. The percentage of positives at each dilution and higher dilutions is calculated from the data of the summary columns. In this case there are 80 per cent positive reactions at 10^{-5} and 20 per cent at 10^{-6} . 50 per cent would then be $30/60$ or 0.50 of the distance from 10^{-6} to 10^{-5} or $10^{-5.5}$ which is equal to 3.16×10^{-6} . The stability of this end-point and the slight effect of chance variation of a single inoculation will readily be appreciated on inspection of Fig. 1. This diagram is constructed by plotting the proportion of positive

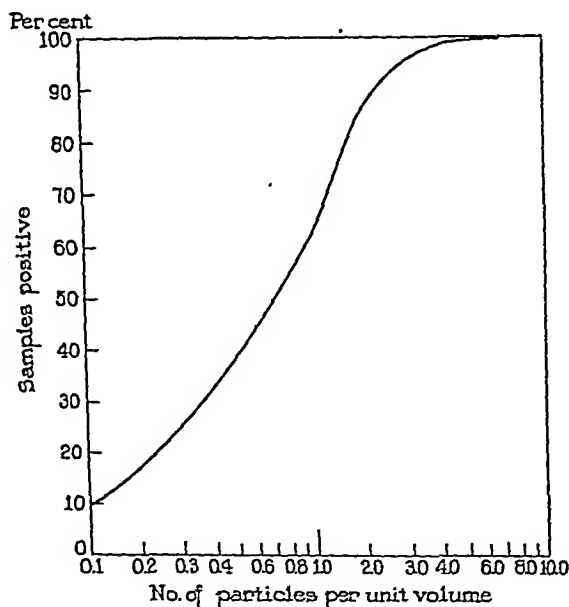


FIG. 1. Probability of obtaining fertile sample, with indicated number of particles per cubic centimeter.

results to be expected against the dilution. It will be seen that when about 50 per cent of the results are positive the curve is quite steep and relatively large variations in the percentage represent relatively minor variations in dilutions. If the end-point is selected at which all of the inoculations could be expected to be positive, it will be seen that relatively minor chance variations will represent relatively large variations in dilutions of material.

In the case of particles representing a relatively small part of the volumes investigated, such as a few bacteria in 1 cc. samples of fluid, the proportion of samples withdrawn which can be expected to contain

at least one organism bears a definite relation to the mean number of organisms in the whole sample. With elementary bodies, however, it has not yet been shown that a single body gives rise to infection, and for that reason it is probably unjustifiable to apply this method of determining the true number of infectious units in the sample. In the present study therefore, the procedure will be adopted of assuming that the number of infectious units contained in a suspension is the reciprocal of the dilution which can be expected to give rise to 50 per cent of positive inoculations on intradermal injection. Thus a suspension giving an end point of $10^{-5.5}$ would be said to contain $10^{5.5}$ or 3.16×10^5 infectious units.

Correlation between Number of Elementary Bodies in a Suspension and Its Infective Titer

With the development of technics by which suspensions of elementary bodies relatively free from other particulate material can be prepared, it became apparent that in general a relation existed between the number of elementary bodies and the infectious units. In order to ascertain whether an exact correlation between these two properties of a suspension exists, the following experiment was carried out.

Experiment 3.—A number of suspensions were prepared as described above which varied widely in their content of elementary bodies. Titration in the rabbit was carried out immediately after preparation, and counts of elementary bodies were made as promptly as possible. The results are presented in Table IV, and in Fig. 2.

On inspection of Fig. 2, there is seen to be a close association between the infectious titer of the suspensions studied and the number of particles which they contained as determined by direct count. This is expressed by the high correlation coefficient of 0.845 ± 0.058 . At the mean of the distribution the ratio of particles to infectious units is that of the logarithms 9.62 to 8.0 (4.19 billion to 100 million). However, the portion of the curve which has been defined is not sufficiently long, nor is its probable error sufficiently small, to warrant assumptions as to its true form. For this reason it is not justifiable to produce the curve to its base, and to state that an infectious unit of virus contains on an average 41.9 elementary bodies. Such a conclusion is not justifiable on the basis of any statistical information obtained to date.

We wish also to emphasize the fact that the correlation table below holds only for the suspensions of elementary bodies prepared in a definite manner from a certain strain (C L) of vaccine virus, the infectivity of which has been determined by intradermal inoculation of rabbits. The manner of titration of vaccine virus is of great importance for there may be a thousandfold difference between the results of dermal and intradermal inoculation. Different strains also vary

TABLE IV

Correlation of Infective Titer of Suspensions with the Number of Elementary Bodies Determined by Direct Count

Suspension No.	No. of elementary bodies per cc.	No. of infectious units per cc.	Log—elementary bodies— x	Log—infectious units— y
1	3.27×10^7	8.60×10^5	7.51	5.93
2	1.18×10^8	6.41×10^5	8.07	5.81
3	9.10×10^8	1.52×10^6	8.96	6.18
4	7.59×10^{10}	5.00×10^8	10.88	8.70
5	2.39×10^9	1.85×10^8	9.38	8.27
6	3.05×10^{11}	1.30×10^{10}	11.48	10.11
7	4.50×10^{10}	8.84×10^9	10.65	9.95
8	1.89×10^{10}	1.32×10^9	10.28	9.12
9	4.30×10^9	2.72×10^9	9.63	9.43
10	2.47×10^9	1.85×10^7	9.39	7.27
11	9.40×10^8	1.85×10^7	8.97	7.27
12	6.29×10^8	1.41×10^7	9.80	7.15
13	1.37×10^{10}	6.81×10^8	10.13	8.83
Mean.....			9.62	8.00
σ			1.183	1.466

$$r = 0.845 \pm 0.058.$$

$$b_1 = 0.682.$$

$$b_2 = 1.042.$$

greatly, and we may not safely infer that findings true of one strain will be true of all others without qualification.

Agglutination Reaction

In the agglutination reaction there is a variation in the apparent titer of the serum which depends on the number of antigenic particles present. This phenomenon, well known in bacteriology, is observed

also in the titration of serum against elementary bodies, and for each suspension, therefore, the dilution must be found which will agglutinate in the presence of the highest dilution of serum. At the same time, it is obvious that when the number of particles present in a suspension is reduced below a certain point, no agglutination will take place. It has been shown that this latter point is governed largely by the surface

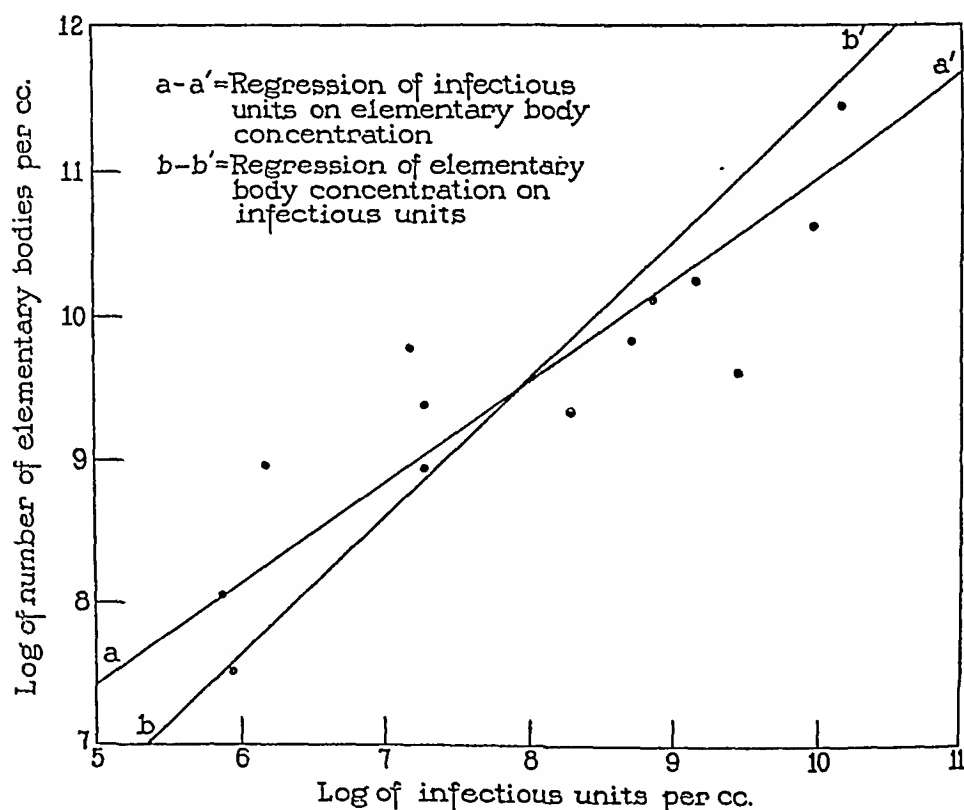


FIG. 2. Correlation of infectious titer of suspensions with the number of elementary bodies determined by direct count.

area of the antigen in relation to its mass, and, in a recent paper, Merrill (7) has collected a number of observations on this subject. In order to obtain more accurate data with regard to the agglutination of elementary bodies, the following experiment was carried out.

Experiment 4.—The optimum dilution of a suspension of elementary bodies for the agglutination test was determined by making several titrations of an immune serum for each of which a different dilution of elementary bodies was used;

the dilutions of elementary body suspensions were selected which showed the maximum titer of agglutination. In the determination of the minimum number of elementary bodies necessary for the production of visible agglutination, titrations of elementary body suspensions were made using several dilutions of serum. The results of several such experiments are given in Table V.

Inspection of Table V reveals that the optimum concentration of elementary bodies for the titration of serum extended over rather a broad range, varying between mean values of log 10.02 (1.05×10^{10}) and log 9.31 (2.04×10^9) particles per cc. The diameter of the elementary body of vaccinia is probably between 125 and 175 m μ (8). If we accept these figures, and apply the calculations outlined by

TABLE V

Concentration of Elementary Bodies Required for Visible Agglutination and Optimum for Serum Titration

Log—No. of elementary bodies per cc. required for visible reaction	Log—largest number of elementary bodies per cc. giving optimum agglutination	Log—least number of elementary bodies per cc. giving optimum agglutination
8.45	10.04	9.43
7.72	10.41	8.66
8.57	9.75	9.15
8.59	9.97	9.67
8.12	10.03	9.67
Mean.....8.29	10.04	9.31

Merrill (7), we learn that visible agglutination is not to be expected when the concentration of elementary bodies falls below log 8.26 particles per cc. It will be seen in Table V that the concentration of elementary bodies below which agglutination did not occur, was on the average log 8.29 particles per cc. Thus the observed and expected findings agreed closely.

Densitometer Measurements

In view of the relation demonstrated to exist between the content of elementary bodies and the infective and agglutinating titers of a suspension, the rapid estimation of the density of a preparation becomes of practical importance. Methods of estimating the density of bacterial suspensions have been described from time to time, one

of the simplest being that of Gates (4), which is based upon the fact that, for a given suspension, the depth of medium required to obscure a wire loop submerged in it is proportional to the number of organisms. In order to determine whether this method is suitable for the estimation of the number of elementary bodies in suspensions, the following experiment was performed.

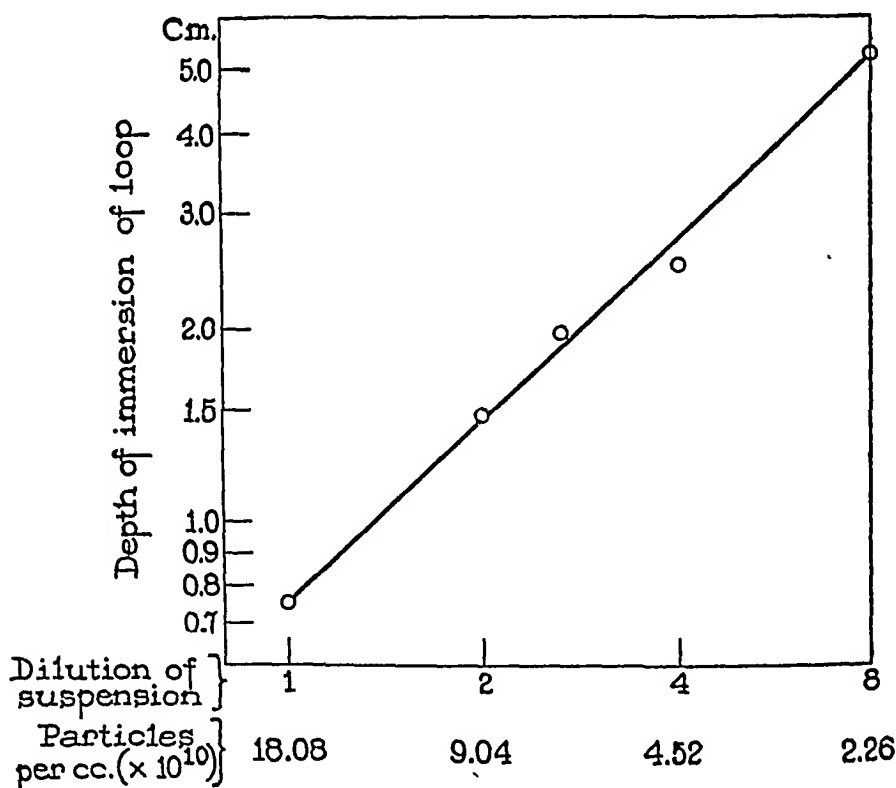


FIG. 3. Correlation of depth of immersion of loop with density of suspension.

Experiment 5.—A heavy suspension of elementary bodies was prepared and 2 cc. were placed in the densitometer tube. The depth to which it was necessary to submerge the wire loop in order to cause it to disappear was observed, several readings being made. The suspension was then diluted progressively with buffer solution and readings were made after each dilution. The number of particles present in the original suspension was determined by direct count.

The results of a typical experiment are presented graphically in Fig. 3. It will be seen that the determined points fall approximately on a straight line, the coefficient of variation of the measurements

being roughly 3 per cent. We have included on this graph the number of elementary bodies per cubic centimeter.

In the original description of the method it was shown that in order to obtain strict proportionality between the number of organisms and the turbidity of the suspension the observed reading must be corrected for an error which is introduced by the transparency and size of the bacteria. In the case of elementary bodies the correction to be applied is evidently within the limits of error of the measurement and may therefore be disregarded. Furthermore, by the use of a logarithmic scale, the actual number of elementary bodies represented may be read off directly once the disappearance depth of the wire loop for a suspension of known concentration has been found. It should be borne in mind, however, that the method is a subjective one, involving a number of important variables. For that reason it must be standardized by each worker for the conditions under which it is to be used, either by counting the number of particles in a suspension the density of which has been determined, or by titrating its infectivity.

SUMMARY AND CONCLUSIONS

Methods have been described by which the number of elementary bodies present in a suspension can be estimated. It has been shown that by means of replicate counts, in which the Petroff-Hausser chamber was used, a high degree of accuracy can be attained. By means of the Gates densitometer, the number of elementary bodies in a suspension can be determined with a coefficient of variation of about 3.0 per cent. A method has been described by which the accuracy of estimation of the infectious titer of a suspension can be increased without greatly enlarging the number of animals employed. This consists of selecting as the end-point that dilution of virus which on intradermal inoculation in a rabbit would lead theoretically to an equal number of positive and negative results. The statistical advantages of this method have been confirmed by the experiences of other laboratories.

By the application of the methods described, there was shown to be a direct correlation between the number of elementary bodies and the number of infectious units of virus present in a given suspension. At the mean of the distribution this ratio is as the logarithms 9.62 to 8.0. To extrapolate this curve, in order to determine the number of elementary bodies present in a single infectious unit, while tempting, is probably not justifiable. It must likewise be remembered that the data given apply to a particular strain of vaccine virus, and that the

number of infectious units has been determined by intradermal inoculation of rabbits. It appears also that this method may be of value in studies of the virulence of different strains of vaccine virus, since by its application one may determine not only the infectious titer of a suspension, but its content of elementary bodies.

In the agglutination reaction it was found that optimum titers of serum were obtained when the test antigen contained from 2.0×10^9 to 1.05×10^{10} elementary bodies per cc. Approximately 1.95×10^8 particles per cc. of suspension were required for the production of visible agglutination.

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THE HEMOCYTOLOGICAL CONSTITUTION OF ADULT MALE RABBITS FROM FIFTEEN STANDARD BREEDS

By ALBERT E. CASEY, M.D., PAUL D. ROSAEN, M.D.,
CH'UAN-K'UEI HU, M.D., AND LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)
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A study of the blood cytology of normal rabbits has been undertaken in this laboratory for some years for the purpose of obtaining precise data which could be used in our investigations on the susceptibility and resistance of the animal host to disease agents (1). The work has included repeated examinations of groups of rabbits over periods of 2 weeks to 2 years, and in addition a large number of animals derived from various sources have been examined one or more times. The information obtained has shown that wide variations in the blood cell formulae of normal rabbits are found (2). Some of the differences could be related to seasonal conditions while others were apparently due to technical causes or to intercurrent disease. It was also found that certain differences in the blood formulae of individual animals could be definitely correlated with the individual reaction to experimental syphilis (3) and to a transplantable neoplasm (4).

This study on blood cytology has been extended in various directions and the influence of such factors as disease (5), breed (6), sex (7), age, certain physiological states, and diet has been investigated. The present paper is the report of observations made on laboratory bred strains of standard varieties of rabbits. The results are not to be interpreted as representing necessarily so called normal or standard blood cell values for these particular breeds but rather are to be considered from the standpoint of genetically related and genetically unrelated individuals. A preliminary report on certain of the results has already been published (6).

Materials and Methods

The results to be reported are based upon an analysis of the blood cytology of 180 rabbits representing fifteen standard breeds (Table I). A detailed description of these breeds may be found in the Catalogue of the American Rabbit and Cavy Breeders Association (8).¹ These fifteen varieties had been propagated in pure line in this laboratory from 3 to 8 years under uniform dietary and housing conditions.

TABLE I
Hemocytological Constitution of Standard Breeds of Rabbits
Data on Material and Methods

Breed	No. of animals	Age		Bleedings		Determinations			
		Mean	Variance of mean			RBC, Hb, and platelets	Total WBC	Total smears	Total white cells counted in smears
		mos.	mos.	days	wks.				
Havana.....	24	7.6	1.27	3.6	2.2	3.8	5.2	6.5	700
Himalayan.....	19	5.1	0.07	1.6	1.3	4.0	4.0	5.9	937
Belgian.....	14	9.8	4.80	3.6	2.4	4.2	6.1	6.6	814
English.....	24	8.4	2.47	3.3	1.8	4.2	5.0	6.4	700
Polish.....	16	8.3	2.48	4.1	2.4	4.1	6.4	6.8	713
Dutch.....	18	9.5	3.55	3.0	2.0	3.5	5.0	6.5	833
Beveren.....	18	8.9	3.28	4.3	3.3	4.7	5.0	6.8	739
Rex.....	13	7.7	1.65	4.0	2.5	4.0	6.1	6.5	715
Chinchilla.....	10	9.6	4.55	4.0	2.0	4.0	5.5	6.0	600
French Silver.....	8	6.3	—	4.0	2.0	4.0	5.5	6.0	600
American Blue.....	6	10.0	—	3.0	1.7	4.0	5.0	5.7	733
New Zealand.....	4	4.7	—	1.0	1.0	4.0	4.0	5.0	1000
Flemish.....	3	10.7	—	1.0	1.0	4.0	4.0	5.0	1000
Gouda.....	2	5.0	—	1.0	1.0	4.0	4.0	5.0	1000
Tan.....	1	4.7	—	3.0	1.0	3.0	6.0	6.0	600
Total.....	180	8.1		3.3	2.1	4.0	5.4	6.3	759

Sex.—Only male rabbits were employed.

Age.—The exact age of each rabbit was known. When the counts extended over more than 1 week the age was recorded as being that of the middle of the

¹ The animals were all derived from the large rabbit breeding colony of Dr. Wade H. Brown which has been maintained at The Rockefeller Institute for some years for the study of constitutional problems.

period; this period was in no instance longer than 1 month. The majority of the animals were from 4 to 10 months old with extremes of 3.4 and 36.0 months, and a mean age for the 180 animals of 8.1 months. With one exception the mean ages of the various breeds were not significantly different. The Himalayan breed was the exception with a mean age of 5.1 months. This breed had no animal younger than 4.2 months or older than 8.0 months at the time of examination (Table I).

Diet.—The diet which was constant throughout the entire period of observation consisted of hay, oats, and a commercial food pellet, with a free access to water. The pellet was rich in vitamins and mineral salts.

Period of Observation.—The observations were begun on Mar. 25, 1931, and were terminated on Nov. 5, 1932. The 180 animals were examined as follows: 14 in March, 1931; 25 in March, 1932; 1 in April, 1931; 41 in April, 1932; 11 in May and June, 1931; 10 in September, 1931; 20 in September, 1932; 44 in October, 1932; 7 in November, 1931; and 7 in November, 1932 (Table II). Three-fourths of the animals were examined in five groups containing 14 to 41 animals per group, and each representing a number of breeds. Of the total number of rabbits, 87 were examined in the spring and 88 in the fall. Only 5 of the 180 rabbits were examined in the summer and winter (June, July, August, December, January, and February). Of the eleven breeds represented by 6 or more animals, six were equally, and five were unequally (Himalayan, English, Beveren, Dutch, and Polish) distributed between spring and fall. This point will be discussed later.

Physical Condition and Housing.—The animals were in excellent physical condition and free from intercurrent disease as far as could be determined by frequent inspection. The observations were all made between 1.5 and 21.0 months prior to the occurrence of an epidemic of rabbit pox, the first cases of which appeared in this colony late in December, 1932 (9). During the 60 days preceding the outbreak only 7 animals were examined. These were distributed as follows: 2 Havana, 1 Belgian, 1 English, 1 Beveren, 1 Chinchilla, and 1 Black and Tan. (Table II). The 7 animals were seemingly healthy, were scattered through six breeds and made only 1/26 of the total number examined. Even had rabbit pox been present in several of the 7 animals no appreciable bias in the results could have occurred.

The animals were not subjected to any other tests at the time of counting. Many of the older animals had been used for breeding. Each animal was kept in an individual cage in a well ventilated room with good lighting.

Hematological Technique.—From one to six blood samples (average 3.3) were obtained from each animal during a period of 1 to 4.5 weeks (average 2.1 weeks). The blood samples were taken between 9 and 12 a.m. and between 1.30 and 4 p.m. Approximately the same number of counts were made on each breed during the morning and afternoon periods. Two individuals alternated in taking the blood from the marginal ear vein, and opposite ears were used for succeeding counts. (Alcohol, 50 per cent, was used to wet the ear before shaving.) Two or three persons made the differential counts using duplicate or triplicate smears and one

person made the red blood cell, the hemoglobin, the platelet, and the total white blood cell determinations. For each animal from 3 to 6 red blood cell, platelet, and hemoglobin estimations were made with the general average of 4.0; 4 to 12 total white cell counts were made with a general average of 5.4; from 5 to 8 blood smears with an average of 6.3. The total number of white blood cells counted on all smears for each animal varied from 500 to 1200 with a general mean of 759

TABLE II

Distribution of Animals According to Periods of Observation

Mean of period	Ha*	H	B	E	Bv	D	P	C	R	S	F	A	Z	G	T	Totals
<i>1931</i>																
Mar. 25.....	0	6	2	3	0	0	0	0	0	0	3	0	0	0	0	14
Apr. 27.....	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
May 19.....	0	0	0	0	0	0	0	0	0	0	0	2	4	0	0	6
June 11.....	0	0	1	0	2	0	0	0	0	0	0	0	0	2	0	5
Sept. 17.....	0	7	0	0	0	3	0	0	0	0	0	0	0	0	0	10
Nov. 18.....	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	7
<i>1932</i>																
Mar. 25.....	6	5	0	8	0	6	0	0	0	0	0	0	0	0	0	25
Apr. 19.....	7	0	4	7	1	0	6	5	5	4	0	2	0	0	0	41
Sept. 28.....	2	0	2	4	2	2	2	4	0	0	0	2	0	0	0	20
Oct. 18.....	6	0	4	0	2	6	8	0	7	3	0	0	0	0	0	36
Oct. 28.....	1	0	0	1	3	1	0	0	1	1	0	0	0	0	0	8
Nov. 3.....	2	0	1	1	1	0	0	1	0	0	0	0	0	0	1	7
Total (12 periods)...	24	19	14	24	18	18	16	10	13	8	3	6	4	2	1	180
Winter.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spring.....	13	12	6	18	1	6	6	5	5	4	3	4	4	0	0	87
Summer.....	0	0	1	0	2	0	0	0	0	0	0	0	0	2	0	5
Fall.....	11	7	7	6	15	12	10	5	8	4	0	2	0	0	1	88

* Ha, Havana; H, Himalayan; B, Belgian; E, English; Bv, Beveren; D, Dutch; P, Polish; C, Chinchilla; R, Rex; S, French Silver; F, Flemish; A, American Blue; Z, New Zealand; G, Gouda; T, Black and Tan.

(Table I). Standardized Trenner automatic pipettes were used. The red blood cells and the platelets were counted by the method of Casey and Helmer (10). The hemoglobin was determined by the Newcomer method, using a Bausch and Lomb hemoglobinometer. Repeated checks with other instruments and by several observers were made. The neutral red supravital technique was employed for the differential white cell counts. Smears were counted in the open laboratory without the use of the hot box, from 1 to 4 hours after their preparation (11).

Statistical Procedures.—All observations of the 14 blood factors (red blood

cells, hemoglobin, platelets, and total white cells, and neutrophils, eosinophils, basophils, lymphocytes, monocytes, both per cubic millimeter of blood and in percent) were averaged for each animal and the means of these values together with the variance of the means were determined for each breed (12). The analysis of the variance and other statistical procedures follow the methods outlined by Fisher (13) and Snedecor (12).

RESULTS

The breed means of each blood factor are summarized in Table III, and the variance of these means (the square of the standard error of the mean) for the eight breeds with the largest representation in Table IV. A general mean for the nine breeds represented by 10 or more animals is presented for each blood factor (Table III), and also for the fifteen breeds (averaging 12 animals per breed) (Table III). Means for the 180 animals without regard to breed means are presented at the foot of this table.²

The most important results, however, concern an analysis to determine whether for the various blood factors the variance between the eight most represented breeds was greater than the variance within these breeds. The results of this analysis expressed by the distribution of F are to be found in Table V. The significance of F (12) is presented by the probability column in this table.³ As shown at the

² The two tables can be used as follows: To determine whether the red blood cell level in the Havana breed was significantly greater than that in the Rex breed, one would proceed in the following manner. First, the difference between 5,730,000 red blood cells per c.mm. in the 37 Havana rabbits and 4,870,000 red cells per c.mm. for the 13 animals in the Rex breed was 860,000 red blood cells. The significance of this difference is calculated by adding the variance of the mean for red cells in the Havana breed of 104 (Table IV) to the variance of 147 for the mean red cell level in the Rex breed giving a value of 251. This latter value is the variance of the difference. The square root of 251 equals 15.8. This is the standard error of the difference, and the difference between the two mean red cell values is then expressed as $860,000 \pm 158,000$ red blood cells. As the difference is more than 5 times the standard error of the difference the Havana breed can be said to have had a significantly higher red blood cell level than the Rex breed. The differences between other blood cell factors can be determined in a similar manner by the use of Tables III and IV.

³ Significance is taken to mean a value which should not be expected to occur by random association of variables more frequently than once in 100 opportunities, and probable significance a value which would not be expected to occur more often than once in 20 opportunities or less often than once in 100 opportunities.

TABLE III
The Hemocytological Constitution of Standard Breeds of Rabbits (180 Healthy Young Adult Males)

Breed	No. of animals	Age mos.	Means of individual means													
			RBC	Hb per cent	P	WBC	N	B	E	L	M	N%	B%	E%	L%	M%
												per cent	per cent	per cent	per cent	per cent
Havana.....	24	7.6	573	73.4	562	653	349	36	7	203	57	53.7	5.6	1.1	31.0	8.5
Himalayan.....	19	5.1	565	67.8	476	707	325	44	12	263	65	46.2	6.6	1.7	36.5	9.3
Belgian.....	14	9.8	532	68.6	527	776	376	68	14	239	77	48.9	9.0	1.9	30.6	9.7
English.....	24	8.4	534	68.1	561	680	372	55	11	181	60	54.1	8.5	1.7	26.7	8.9
Polish.....	16	8.3	562	73.9	577	865	375	32	9	378	71	43.5	3.8	1.1	43.5	8.1
Dutch.....	18	9.5	526	71.8	635	733	398	37	11	214	71	53.6	5.3	1.6	29.9	9.6
Beveren.....	18	8.9	520	68.1	531	858	374	69	13	310	84	43.8	8.5	1.7	35.6	10.1
Chinchilla.....	10	9.6	497	62.3	548	905	429	53	16	325	79	47.6	6.2	1.7	36.1	8.4
Rex.....	13	7.7	487	63.6	595	782	377	62	7	262	74	48.1	7.9	0.9	33.9	9.2
Mean breeds.....			533	68.6	557	773	375	51	11.1	264	71	48.8	6.8	1.49	33.8	9.1
French Silver.....	8	6.3	498	64.9	607	816	389	37	5	267	118	47.8	4.6	0.7	32.3	14.6
American Blue.....	6	10.0	520	67.3	713	841	463	66	12	200	101	55.1	8.0	1.5	24.0	11.6
New Zealand.....	4	4.7	562	69.8	798	989	436	64	11	366	113	43.4	6.4	1.2	37.5	11.5
Flemish.....	3	10.7	555	69.1	388	802	374	47	26	270	85	46.5	5.9	3.4	33.6	10.6
Gouda.....	2	5.0	598	74.8	462	936	565	48	37	181	108	59.3	5.2	4.3	19.8	11.6
Tan.....	1	4.7	555	76.3	690	648	281	30	10	296	31	43.5	4.5	1.6	45.7	4.8
Mean breeds.....	12.0	7.8	539	69.3	578	799	392	50	13.4	264	80	49.0	6.4	1.74	33.1	9.8
Mean individuals.....	180	8.1	537	69.1	566	769	378	50	11.1	255	74	49.4	6.7	1.51	32.9	9.5

RBC, red blood cells (0,000 omitted); Hb, hemoglobin (Newcomer); P, blood platelets (000 omitted); WBC, N, B, E, L, M, total white blood cells, neutrophils, basophiles, eosinophiles, and monocytes (0 omitted); N%, B%, E%, L%, M%, relative numbers of the white blood cells.

TABLE IV
The Hemocytological Constitution of Standard Breeds of Rabbits

Breed	No. of animals	Age mos.	Variance of the mean (the square of the standard error of the mean)										
			RBC	Hb	P	WBC	N	B	E	L	M	N%	D%
			per cent	per cent								per cent	per cent
Havana.....	24	1.27	104	1.49	322	853	316	13	0.5	140	30	2.21	0.22
Himalaynn.....	19	0.07	50	1.03	296	1950	606	15	2.0	526	21	3.59	0.35
Belgian.....	14	4.80	144	0.43	519	2180	663	33	5.5	420	81	2.83	0.50
English.....	24	2.47	104	1.37	251	886	521	17	0.9	174	19	2.65	0.50
Polish.....	16	2.48	111	1.78	635	1040	479	11	1.4	596	82	3.07	0.19
Dutch.....	18	3.55	122	1.32	1835	1237	1039	12	1.1	286	54	5.71	0.20
Beveren.....	18	3.28	163	2.06	329	2497	659	60	6.8	995	41	5.11	0.99
Rex.....	13	1.65	147	3.91	503	981	576	72	0.7	189	51	3.85	0.50
Total.....	180	0.04	17	0.23	77	179	67	3	0.5	59	6	0.45	0.06

TABLE V
Hemocytological Constitution of Standard Breeds of Rabbits
*Analysis of the Variance**

Standard deviation	No. of animals	Age mos.	RBC	Hb	P	WBC	N	B	E	L	M	N%	D%	E%	L%	M%
				per cent								per cent	per cent	per cent	per cent	per cent
			Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
Total.....	145	6.5	52	6.1	108	174	104	25	6.6	102	28	9.0	3.3	0.8	8.8	2.8
Within breeds.....	138	6.5	46	5.4	101	161	105	22	6.2	85	28	8.1	2.8	0.8	7.5	2.8
Between breeds.....	7	6.3	116	14.0	203	337	95	62	11.6	273	39	19.5	8.6	1.5	22.1	2.6
F.....	1.01	6.7	6.7	4.0	4.0	4.4	1.2	8.0	3.5	10.3	1.9	5.8	9.4	3.5	8.7	1.2
P.....	—	0.01	0.01	0.01	0.01	0.01	—	0.01	0.01	0.01	—	0.01	0.01	0.01	0.01	—
Significant.....	—	—	Sig.	Sig.	Sig.	Sig.	—	Sig.	Sig.	Sig.	—	Sig.	Sig.	Sig.	Sig.	—

* Limited to the eight most represented breeds.

bottom of Table IV the breeds were found to differ significantly among themselves with respect to red blood cells, hemoglobin, blood platelets, total white blood cells, to basophiles, eosinophiles, and lymphocytes per cubic millimeter and in per cent, and to neutrophiles in per cent. No significant differences in the monocytes per cubic millimeter or in per cent, or in the neutrophiles per cubic millimeter, or in the age of the various breeds were detected.

DISCUSSION

The present study of the hematological constitution of rabbits carried out on 180 male animals of known age and breed is unique in several respects. Earlier studies of our own (14) as well as those of other workers were not corrected for breed and age (15-17). The present investigation concerns mean blood levels rather than random blood cell determinations, uncorrected for technical error. The extent of the variations are, therefore, much less than that reported by ourselves or others. The animals were well adapted to cage life and were perhaps better nourished and healthier than the mongrels studied earlier. It will be seen that the mean red blood cell values for male rabbits do not significantly differ from the mean red blood cell values for human males as published by Price-Jones (18), Osgood (19), Wintrobe (20), McGeorge (21). The mean value compiled from the four authors for the 323 healthy young men, averaging about 25 years of age, was 5,433,000 red blood cells per c.mm. of blood. This differs from the 5,370,000 red blood cells obtained for rabbits by $63,000 \pm 45,300$, and since the difference is less than twice the standard error, it is not significant. It seems quite remarkable that two species so widely different as to size and many other characteristics including hemoglobin should be alike in this respect. Since the samples of both species examined are comparatively large the results are probably fairly accurate.

The present results show significant variation in the blood cell levels between the breeds for red blood cells, hemoglobin, basophiles, lymphocytes, eosinophiles, platelets, and white blood cells. This variation was significantly greater between the breeds than within the breeds. This would seem to indicate that in the rabbit genetically related individuals have similar blood formulae and genetically unre-

lated individuals dissimilar blood formulae. This conclusion is tenable only if it can be shown that no other variables have biased the results. A consideration of such factors as age, sex, season, nutrition, housing, intercurrent disease, hematological technique, and stature which might influence the results will, therefore, be made.

Sex and Age.—Sex played no part because the animals were males. Since the work of Sabin (22) has shown that the blood formula of rabbits changes from infancy to maturity some consideration of age had to be made. All breeds were of comparable mean age with the exception of the Himalayan in which there were no animals older than 8 or younger than 4 months. The age of this breed was probably significantly less than that of most of the other breeds ($P = 0.05$). However, the remaining breeds which show no difference as to age show wide variations in their formulae. In the case of the eight breeds with 13 or more animals (Himalayan included) the variance between the breeds due to age was not different from the variance within the breeds ($F = 1.01$; Table V). Furthermore, the blood formula of the Himalayan breed is that of adults (note the lymphocyte level) and not that of young or immature rabbits. It must be concluded, therefore, that neither age nor sex will explain the breed differences detected. Again an analysis of the material in various age periods revealed no delayed or irregular maturation on the part of any breed to account for its blood formula; nor was the variance between the various age periods significantly greater than the variance within the various age periods for any blood factor. A subsequent publication will discuss the age of the animals in relation to their blood formula (23).

Housing and Nutrition.—All the rabbits were subjected to the same housing and nutritional conditions and each breed had been propagated in pure line in the same laboratory for periods of 3 to 8 years. However, it is conceivable and there is evidence to support the conception that a diet and indoor life which is suitable for one breed may not be suitable for other breeds. Large animals might have had a blood formula which differed from that of small or medium sized animals in cages of similar size. However, there was a wide variation in the blood picture among such small or medium sized animals as the Dutch, Himalayan, Polish, Havana, and English where the confine-

TABLE VI
Hemocytological Constitution of Standard Breeds of Rabbits
Difference between Large and Small Breeds

	No. of animals	RBC	Hb	P	WBC	N	B	E	L	M	N%	B%	E%	L%	M%
Large and Heavy Breeds															
Mean.....	53	525	67.8	574	832	391	62	13	274	91	47.1	7.7	1.6	32.5	11.0
Variance of mean....		8	0.8	18	26	14	4	1	15	5	1.1	0.5	0.2	1.1	0.5
Small and Light Breeds															
Mean.....	80	559	71.9	560	733	364	38	10	255	66	49.9	5.4	1.4	34.5	8.9
Variance of mean....		5	0.6	14	36	13	2	1	12	3	1.1	0.3	0.1	1.1	0.3
Differences															
Difference.....	—	34	4.1	14	99	27	24	3	19	25	2.8	2.3	2.0	2.1	2.1
σ difference.....		± 10	± 1.0	± 23	± 25	± 19	± 4	± 2	± 19	± 6	± 1.6	± 0.5	± 0.2	± 1.6	± 0.6
<i>t</i>		3.4	4.0	—	3.8	—	5.7	—	—	4.1	—	4.2	—	—	3.3
<i>P</i>		0.01	0.01	—	0.01	—	0.01	—	—	0.01	—	0.01	—	—	0.01

ment was comparable. Each animal was kept in an individual cage. Possible hereditary variations among the breeds, such as peculiar dietary requirements and differences in adaptability to cage life, might have been concomitant with, rather than the cause of the variations in the blood formula. In this connection the material tabulated in Table VI is of interest. The breeds were divided into heavy, intermediate, and light groups according to weight. The Flemish, New Zealand, American Blue, Blue Beveren, Belgian, and French Silver animals, 53 in all, were grouped in the heavy class. The Rex, English, and Chinchilla animals were grouped as intermediate. The Gouda, Havana, Polish, Himalayan, Tan, and Dutch animals, 80 in all, were grouped in the light weight class. It will be seen that the heavy animals had significantly lower red blood cells and hemoglobin, and higher white blood cells, basophiles, and monocytes than the light weight animals. That such differences are not associated entirely with weight or build is indicated by the formula in the Polish breed which had the highest white blood cell level, and in the French Silver which had a low basophile level. The information at hand does not indicate whether this difference in blood formula is due to body build or weight, or whether animals in breeds having similar weights were derived genetically from a similar stock. A third factor or factors might be responsible for both weight and blood formula. Studies of breed differences in blood formulae in other animal species might clear up this point. In any event the results obtained emphasize the genetic constitution of the blood formula. The significant variations among the breeds as to the blood platelets, the eosinophils, and the lymphocytes, and the neutrophils in per cent were not associated with differences in body weight or size.

Intercurrent Disease.—Studies in this laboratory have shown wide variations in susceptibility of the various breeds to experimental and spontaneous diseases. The view that the variations in the blood formulae as here reported were in reality associated with unnoticed intercurrent disease might be tenable if the clinical observations had been desultory and the blood variations of a simple order. However, the former were systematic and the latter so diverse and so often without relation to each other that an explanation based on disease conditions does not seem plausible or likely. The animals were healthy in appearance, showed no loss of weight, and there was no clinical evidence

of such spontaneous conditions as snuffles, ear canker, gastro-enteritis, or other malady. In this connection it may be pointed out that in previous studies no demonstrable lesions were found at postmortem examination to account for the variations in blood formulae in individual animals.

Technical Error.—From studies of the technical error in blood counting⁴ it can safely be said that the differences between breeds could not be explained on this basis. The error in each breed mean due to technique alone is negligible. The time of the day was not a factor since the animals were so alternated that approximately the same number of each breed were counted in comparable periods.

Season.—Investigations in this and in other laboratories have shown wide variations in the blood picture for different seasons of the year. The effect of season was considered in planning the present experiment and no observations were made in winter and only five in summer. The animals were equally divided between the two seasons calculated to show the fewest differences, 87 in the spring and 88 in the fall. Although for most of the breeds the same number of animals were counted in one season as in the other, the variance between breeds as to the month of the year in which counts were made was significantly different from the variance within breeds ($z = 0.786$; $P = 0.01$ —, significant). This was because the Beveren, Dutch, Polish, and Rex breeds were largely examined in the fall whereas the Himalayan, English, Flemish, American Blue, and New Zealand breeds were largely examined in the spring (Table II). However, the following tabulation shows that for every blood factor except the red blood cells and the blood platelets there was no significant difference between the spring and fall levels.

	Animals	Breeds	R	H	P	W	N	B	E	L	M
Spring	87	13	548	69.6	585	751	377	50	10	242	70
Fall	88	12	524	68.2	550	775	374	49	11	265	75
Difference	—	—	24	1.4	35	24	3	1	1	23	5
σ difference	—	—	± 8	± 1.0	± 18	± 27	± 17	± 4	± 1	± 16	± 5
t	—	—	2.8	—	1.97	—	—	—	—	—	—
P	—	—	0.01	—	0.05	—	—	—	—	—	—

⁴ Casey, A. E., Rosahn, P. D., Hu, C. K., and Pearce, L., unpublished material.

For the hemoglobin and the total and individual white blood cells, therefore, there was no bias in the results which could be ascribed to season, and no correction was necessary. In the case of the red blood cells and the blood platelets, it seemed desirable to correct for seasonal variation. This was done by adding 236,000 (the seasonal disparity) to each fall red blood cell value, and 35,300 to each fall blood platelet value. A series of new mean red blood cell and blood platelet values were obtained for each breed which represented the theoretical spring level for all 180 animals. A small correction for the five summer values was also made. The following tabulation shows the corrected red blood cell and blood platelet values, and the direction and amount of change. The variation between the breeds was not diminished by the correction for season, and its significance was not affected either

	Red blood cells	Change	Platelets	Change
Havana.....	583	+10	578	+16
Himalayan.....	574	+9	489	+13
Polish.....	577	+15	599	+22
Belgian.....	542	+10	550	+23
English.....	540	+6	569	+8
Beveren.....	538	+18	568	+37
Dutch.....	542	+16	659	+24
Rex.....	501	+24	617	+22
Chinchilla.....	509	+12	566	+18
Silver.....	510	+12	624	+17

for the red blood cells ($F = 5.6$, $P = 0.01 -$), or for the blood platelets ($F = 4.4$, $P = 0.01 -$). Therefore, although the animals in the fifteen breeds were not distributed evenly between spring and fall these seasonal differences played no part in the variance between the breeds for any blood factor. Furthermore, the results on one large group of 40 animals consisting of various breeds studied during the month of April, 1932, are of special interest (Table VII).

The group comprised 7 Havana, 6 Polish, 4 Belgian, 5 Rex, 5 Chinchilla, 7 English, 2 American Blue, and 4 French Silver animals. There was no significant variation in the ages of the 40 animals. These rabbits were counted in 4 different weeks during this month, and an unusually large number of observations made on each animal. The time of the day was held constant by counting each animal once

TABLE VII
Hemocytological Constitution of 40 Young Adult Male Rabbits
Series of April, 1932

Breed	No. of animals	Age <i>mos.</i>	RBC	Hb <i>per cent</i>	P	WBC	N	B	E	L	M
Havana.....	7	6.6	558	71.2	605	655	359	43	7	184	62
Polish.....	6	4.7	557	72.8	604	990	419	26	9	443	94
Belgian.....	4	6.1	524	67.9	527	886	436	75	8	279	89
Rex.....	5	5.4	480	61.3	596	772	332	79	8	278	75
Chinchilla.....	5	4.3	506	63.4	606	811	374	55	12	309	60
English.....	7	5.4	514	64.4	625	808	489	50	12	185	72
American Blue.....	2	5.3	488	66.3	828	870	509	60	14	215	74
French Silver.....	4	6.0	544	72.5	625	786	352	52	3	282	98
Mean.....	(40)	5.5	525	67.6	613	813	404	53	9	271	77
Mean square											
1. Total.....	556		2649	3723				647	27	12,099	
2. Between breeds.....	316		4392	10,138				1484	44	42,425	
3. Within breeds.....	609		2268	2320				464	23	5465	
F.....	1.9		1.9	4.4				3.2	1.9	7.8	
P.....	0.05		0.05	0.01				0.01	0.05	0.01	
	—		—	Sig.				Sig.	—	Sig.	

during the period between 9 and 10.30 a.m., 10.30 and 12 a.m., 1.30 and 3 p.m., and 3 and 4.30 p.m. In order to do this, the order of counting was changed every week. Individuals from the various breeds were alternated so that the animals in a given breed were not examined consecutively. The factors of time of day, and of week, month, and year were thus held constant. Nevertheless, with only a few animals represented per breed the variance between the breeds was significantly greater than the variance within the breeds for hemoglobin ($F = 4.4$, $P = 0.01 -$), for basophiles ($F = 3.2$, $P = 0.01$), and for lymphocytes ($F = 7.8$, $P = 0.01 -$). Hemoglobin, basophiles, and lymphocytes showed the greatest variation between the breeds in the larger series as well. It is possible that significant breed differences for red blood cells, platelets, and white blood cells would also have been found in this group had there been a larger number of animals. The results on this small group offer other conclusive evidence, therefore, that the variance between the breeds was not due to season.

Breed Relationships.—The exact origin of most of the standard breeds of rabbits is unknown or uncertain, and considerable outcrossing has been practiced from time to time to maintain the vigor of the stock. Although such outcrossing had not been carried out in this laboratory among the breed lines reported in this paper, there is no certainty as to how much this was done before the stocks were brought into the laboratory. There is no certainty that the sample animals for any breed have the same hemocytological constitution as other samples of the same breed elsewhere. It is known that the Havana line used is related to the Dutch in body build, size, and disposition; also inbreeding occasionally demonstrates Dutch markings in pure Havana rabbits. It is also known that the English are in some way related to the Belgians, both being unique in having a greyhound type of body build and similar dispositions. It seemed desirable, therefore, to determine whether or not the blood formulae of related breeds are similar. This was approached indirectly by determining whether related breeds had fewer blood differences than unrelated breeds. This was the case. The Havana blood formula resembled the blood formula of the Dutch more closely than that of any of the other breeds adequately represented, that is, there were fewer signifi-

cant differences. The same was true for the English and the Belgian. Two distinctive breeds such as the Polish and the Himalayan which are unlike any of the other breeds or each other had blood formulae which were also distinctive and unlike that of other breeds. A similar comparison for the fur breeds such as the Cinchilla, Rex, and Beveren is unsatisfactory since coat color or texture may be introduced into various breeds. Thus one may have a Dutch animal with a Rex coat. Analogies as to the blood formulae of these breeds are perhaps unjustified. The Beveren, for example, was found to have a blood formula very similar to that of the Belgian, and although both are large animals and of common Flemish origin no knowledge of interbreeding is available. Finally it should be pointed out that the blood factors observed were but a small sample of the constitutional factors which might have been chosen. The fact that two breeds are alike in a few blood factors does not indicate that they are necessarily alike in other constitutional factors. Of significance, therefore, is the fact that breeds known to be related in genetic origin were also found to have related blood pictures. Certain peculiarities of the breed formulae may be discussed in a subsequent paper.

SUMMARY AND CONCLUSION

A study of the red blood cells, hemoglobin, blood platelets, and the total and individual white blood cells was made on 180 male rabbits of known age and representing fifteen standard breeds. An attempt was made to eliminate or hold constant such variables as age, sex, season, time of examination, technical errors, food, housing, and disease. The mean, variance of the mean, and standard deviation were calculated for each breed sample and for the group as a whole. An analysis of the variance showed that the variation between the breed samples was significantly greater than the variation within the breed samples for the red blood cells, hemoglobin, blood platelets, total white blood cells, basophiles, eosinophiles, and lymphocytes per cubic millimeter and in per cent and the neutrophiles in per cent. No significant variations were detected in the monocytes except when the breeds were divided into heavy and light breeds. No variation in the neutrophiles per cubic millimeter was detected; a large number of the breeds had exactly the same mean neutrophile level. Characteristic

blood formulae were found for the various breed samples having an adequate numerical representation. It was concluded that the varying blood formulae could not be explained on any other, except an hereditary (genetic) basis.

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NITROGEN, POTASSIUM, SODIUM, AND CHLORINE METABOLISM IN RICKETS, WITH SPECIAL REFERENCE TO BILIARY FISTULA RICKETS IN PUPPIES*

BY WALTER HEYMANN, M.D.

(From the Babies and Childrens Hospital, and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland)

(Received for publication, June 8, 1936)

Pavlov (1) discovered in 1904 that biliary fistula in dogs may lead to definite changes in the osseous system. On the basis of studies (2) on this subject which were published in 1932, we concluded that this bone disease could be considered rachitic provided the biliary fistula was performed in puppies from 6 to 7 weeks of age which had been deprived of vitamin D and sunlight from birth on throughout the period of the experiment. It is important, furthermore, that a certain rate of growth be maintained for from 4 to 6 weeks after the operation, so that the rachitic bone lesions may become manifest.

The foregoing conclusion was reached from the following facts: presence of hypophosphatemia; impaired phosphorus and calcium balance; bone changes identified as rachitic in nature by chemical, histological, and roentgenological examination; spontaneous occurrence of tetanic convulsions in one dog accompanied by hypocalcemia and increased mechanical and electrical excitability; and, finally, the curative effect of vitamin D.

In spite of this evidence which, to a great extent, was in favor of the diagnosis of rickets, there was one chief reason for doubting whether this condition might be compared, from the point of view of the metabolism, with rickets that occurs spontaneously in infants or with rickets that is induced by diet in rats; namely, the inhibited weight curve of the puppies which had been operated on. It was shown that the animals in which the gall bladder fistula had been established gained in weight for from 4 to 6 weeks after the operation, al-

* These studies were made partly in the Universitätskinderklinik, Freiburg i. Br., Germany.

The data presented in Tables I and IV show that the gall bladder fistula puppies had a distinctly decreased retention for nitrogen, potassium, and chlorine, as compared with the control group, whereas no difference was found in the sodium balance. The extremely low sodium content of the dog biscuits might have caused the decidedly negative sodium balance found even in the control dogs; doubt is consequently cast on an interpretation of the increased sodium output in the rachitic animals.

The loss of nitrogen, potassium, and chlorine must be explained to a great extent, according to the results obtained, by an increased excretion in the urine. To consider these findings the result of an interference in intestinal absorption is, therefore, out of the question. Even the loss of calcium and phosphates, data for which have been reported in detail in a previous paper (2), cannot be traced to diminished absorption, because it was found that the ratio of phosphates in the stool and urine did not show the increase that it is a well known characteristic of the usual disturbed balance in rickets.

These results point directly to the assumption that, in spite of the fully developed rachitic picture in the gall bladder fistula dogs, the disease observed here must be considered distinctly different from that found in infantile rickets, chiefly because nothing is known as yet about a disturbed balance in infantile rickets concerning other substances than calcium and phosphate. The work done by Schloss (3) in 1916 has been the basis for this assumption. He found that the metabolism of nitrogen, potassium, and sodium in infants suffering from rickets was entirely normal. His studies, however, do not seem to have been repeated, in spite of the fact that his results are of primary importance to the pathogenetic conception of the rachitic state.

Nitrogen, Potassium, Sodium, and Chlorine Metabolism in Rickets and during the Healing of Rickets in Infants

In view of the fact that the results of the experiments on dogs, just reported, differ greatly from those stated by Schloss to have been found in infants, it was thought worth while to investigate the balance of these substances in rachitic infants and in rachitic rats. It is also true that diagnostic and therapeutic methods have become much more accurate since 1916.

Another reason for these further studies was the fact that it is still widely assumed that rachitic hypophosphatemia may be explained by a lack of intestinal absorption of calcium and phosphates (8). There are, however, so far as infantile rickets is concerned, many data (9) which cannot be brought into accord with this theory. The increased excretion of calcium and phosphates in the feces in rickets might just as well be attributed to increased excretion through the intestinal wall. The absorption theory would, on the other hand, be greatly strengthened if one were to find in rickets an increased fecal output also of substances that are not subject to reexcretion through the intestines, for instance, nitrogen, potassium, sodium, and chlorine.

The metabolism of nitrogen, potassium, and chlorine was studied in four infants in periods of 6 days,—in three of these infants while they were ill with rickets and during the process of healing brought about by administration of vitamin D (see Table II). In order to keep conditions comparable during both the rachitic and the healing periods, the same food was given throughout. Infant Me was fed half milk, half gruel, with 5 per cent of dextrimaltose added, while infants Be, We, and Toz were fed half whole, half skimmed milk, with 6 to 8 per cent karo sugar added. Charcoal was used as marker; stool and urine specimens were collected quantitatively and saved in the refrigerator in two parts after having been acidified or alkalinized with H_2SO_4 or NaOH , respectively. Apart from the rickets, the infants were healthy, and had good, formed stools, with the exception of infant We in the rachitic period, when he developed loose and pasty stools. These values, therefore, are excluded from the calculation of averages (Table IV).

No difference was found for the nitrogen and potassium balances in the two groups, exceeding the possibility of experimental error. Considering the average values, there seems to be an increased output of chlorine in the rachitic infants, which, however, does not prove constant when compared in the same child in each group. Average values obtained in a small group may not be reliable enough to be the basis for any further conclusion. The distribution of nitrogen, potassium, and chlorine in stool and urine is practically identical in both groups. This is further evidence of the accidental nature of the chlorine balance in the rachitic infants.

The conclusion to be drawn from these experiments must consequently be that, in agreement with the results of Schloss (3), the metabolism of nitrogen, potassium, and chlorine in infants ill with rickets must be considered entirely normal. The sodium balance could be

Nitrogen, Potassium, Sodium, and Chlorine Metabolism in I

Name	Age	Start and end of metabolism period	Body weight at		Food	Daily treatment	Degree of rickets by x-ray	Ca	
			Start	End				Start	E
	mos.	1933	gm.	gm.				mg. per 100 cc.	mg. 100
Me	4½	Apr. 5 " 12	5510	5300	1:1, 5% 800 cc.	—	++	12.6	9.
Be	11	1935 May 25 " 31	7650	7925	No. 207, 6% 1000 cc.	—	++	7.2	7.
We	7	June 15 " 20	7025	7265	No. 207, 8% 1000 cc.	—	+	—	8.1
F									
Me	4¾	1933 Apr. 18 " 25	5420	5520	1:1, 7% 800 cc.	Vigantol 30-60 units	Some healing	—	9.8
Be	12	1935 June 22 " 28	7600	7900	No. 207, 8% 1000 cc.	Cod liver oil 0.4 gm.	" "	—	9.5
We	7½	July 2 " 9	7310	7750	No. 207, 8% 1000 cc.	" "	" "	9.7	9.7
Toz	9½	Apr. 30 May 6	9605	10,075	No. 207, 8% 1000 cc.	Spontaneous	" "	11.6	10.2

* Done July 15, 1935.

WALTER HEYMANN

with Rickets and in Four Infants during the First Healing Period

Rickets and in Four Infants during the First Healing Period											
Intake				Excretion in							
				Urine				Stool			
N	K	Na	Cl	N	K	Na	Cl	N	K	Na	Cl
g.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
13.858	2.72	0.217	3.635	10.104	2.848	0.326	2.421	0.957	0.162	0.0582	0.042
9.881	10.61	—	7.67	20.3	23.39	—	6.65	3.39	0.543	—	0.122
23.211	8.83	—	6.063	12.0	13.8	—	2.97	5.04	0.956	—	2.94
iod											
14.175	3.78	0.221	3.718	10.99	2.94	0.367	4.51	1.033	0.176	0.0542	0.041
33.351	12.57	—	8.89	19.88	34.68	—	1.97	4.02	0.746	—	0.268
35.536	13.42	—	9.25	16.5	16.18	—	7.63	3.06	0.316	—	0.195
34.85	12.37	—	7.77	23.41	12.42	—	6.98	3.74	0.430	—	0.202

studied in only one case, and the results were in accord with the findings for nitrogen, potassium, and chlorine.

Nitrogen, Potassium, Sodium, and Chlorine Metabolism in Rickets and during the Healing of Rickets in Rats

The results obtained from the studies made on infants, reported above, were confirmed by the following investigation of the nitrogen, potassium, sodium, and chlorine metabolism in seventeen rats (see Table III).

Eleven rats were studied while they were in a rachitic condition. The remaining six were given 10 prophylactic units of vigantol daily,² beginning 4 days before the metabolism period started. At the end of the 8 day metabolism period, the bones of these six rats were almost completely healed, as shown by roentgenogram. The rats of both the rachitic and the healing groups were weaned at the age of 3 weeks, and from this time on were fed McCollum's diet 3143. The metabolism cages were built according to Schultzer (10). Carmine was used as marker.

It may be seen from Tables III and IV that the balance and distribution of nitrogen, potassium, sodium, and chlorine in stool and urine were very much the same in both groups, whether or not the rickets had been cured.

COMMENT

The results of the three experiments described lead to the conclusion that the disease which developed in puppies deprived of vitamin D and sunlight for from 4 to 8 weeks after a gall bladder fistula had been established must be considered different in nature from infantile rickets or from rickets experimentally induced in rats. So far as the bone lesions are concerned, which in all three cases developed because of a diminished content of inorganic phosphate in the blood, we are dealing with rickets in the gall bladder fistula dogs as well. This has been reported in detail in a previous paper (2). The main distinction, however, between rickets induced by biliary fistula and other forms of rickets has been found to lie in an alteration of the entire metabolism in the former, such as is by no means present in infantile rickets or in

² One drop of vigantol to 20 cc. of olive oil. One drop of this solution was used and administered by pipette daily to all rats except Nos. 24 and 25 which received this dose every 2nd day.

TABLE III
Nitrogen, Potassium, Sodium, and Chlorine Metabolism in Rachitic Rats and in Rats Given Rachitic Therapy

Rat No.	Time in metabolism period	Food	Treatment	Degree of rickets by x-ray	Intake				Excretion in							
					N	K	Na	Cl	Urine				Stool			
									N	K	Na	Cl	N	K	Na	Cl

Period of Rickets																
days	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0	6	31.28	—	—	1109	64.5	—	247.3	558	33.9	—	167.3	225	2.2	—	13.5
1	6	36.2	—	—	1220	56.5	—	298	544	31.5	—	150	293	11.2	—	21.5
2	6	37.2	—	—	1258	58.7	—	309	804	57.1	—	217	214	4.5	—	10.3
3	6	29.45	—	—	985	46	—	242	677	38.4	—	177.5	156.7	3.08	—	7.26
9	8	54.2	—	—	1930	75.4	571	476	1158	78	132	344	251	7.24	32	13.95
10	8	62.3	—	—	2220	86.6	657	547	1213	86.6	171	385	354	20.4	17.3	18.9
11	8	53.7	—	—	1912	74.6	565	472	1110	61.2	155.8	352	251.8	2.48	29	7.8
13	8	35.8	—	—	1275	49.8	378	314.5	561	58.4	102.8	147.5	118	12.8	19.1	12.8
14	8	48.5	—	—	1727	—	511	426	785	—	149.3	247.8	172.8	—	19.6	14.9
15	8	57.8	—	—	1846	—	545	455	890	—	148	245	244	—	20.3	20.3
16	8	37	—	—	1318	—	390	325	553	—	82.6	153	240.2	—	30.6	20.5

Healing Period for Rats Given Rachitic Therapy																
days	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
18	8	43.7	Vigantol* 1 drop daily	0	1529	—	—	316	758	—	—	228	178.7	—	—	5.25
19	8	36.1	" "	0	1262	77	218	261	555	48.3	80.1	134.5	172.5	6.45	9.5	13.5
21	8	26.3	" "	0	921	56.2	158.5	190	499.5	42.2	57.7	95.8	133.2	4.07	6.5	4.4
22	8	40.5	" "	0	1417	—	244	292.2	508	—	71.4	128.2	204	—	7.3	10.62
24	8	39.8	Vigantol* 1 drop every 2nd day	0	1391	85	240	287.5	1056	77.1	122.4	177	234.8	2.95	6.18	8.3
25	8	42.3	" "	0	1481	90.3	255	306	1572	76.7	128	233.8	199.3	3.12	7.56	11.7

* Detailed data concerning the vigantol medication are given in the text.

TABLE IV
Summary of Average Values Obtained from Data in Tables I, II, and III

	Urine				Stool				Total			
	N	K	Na	Cl	N	K	Na	Cl	N	K	Na	Cl
Puppies:												
With gall bladder fistula and rickets:												
Excretion of intake, * per cent.....	72.0	2230.0	3710.0	148.0	32.0	156.0	4270.0	10.3	104.1	2386.0	7980.0	158.3
Excretion of total output, per cent.....	70.7	88.6	34.1	93.7	29.2	11.3	66.5	6.0				
Without rickets:												
Excretion of intake, * per cent.....	38.0	1120.0	2120.0	91.5	25.0	237.0	3640.0	18.2	62.8	1367.0	5772.0	109.6
Excretion of total output, per cent.....	58.7	82.6	38.2	84.3	41.2	17.4	61.8	15.7				
Infants:												
During period of rickets:												
Excretion of intake, per cent.....	88.6	96.2		98.3	11.4	3.8		1.6	77.5	167.6		91.1
Excretion of total output, per cent.....												
During first healing period:												
Excretion of intake, per cent.....	84.8	96.7		95.5	15.1	4.3		4.5	72.8	158.0		71.1
Excretion of total output, per cent.....												
Rats:												
During period of rickets:												
Excretion of intake, per cent.....	76.4	85.0	85.0	93.5	23.6	12.0	15.0	6.5	68.2	100.0	31.0	66.0
Excretion of total output, per cent.....												
During healing period:												
Excretion of intake, per cent.....	79.7	93.0	92.0	94.0	20.3	7.0	8.0	6.0	75.0	84.0	44.0	62.5
Excretion of total output, per cent.....												

* Dogs 5 and 3 received, during the metabolism period, dog biscuit from lots in which the K and Na content differed greatly from that in the dog biscuit given to dog 1. Consequently the above values for K and Na include only the data from dogs 5 and 3, for which the intakes of K and Na were comparable.

experimental rickets in the rat. The disturbance of the metabolism in biliary fistula rickets in dogs led to an inhibited gain in weight and, furthermore, to an increased excretion not only of calcium and phosphates but also of nitrogen, potassium, sodium, and chlorine. The infants ill with rickets, on the other hand—and this was found to be true also of the rachitic rats—showed an isolated disturbance in the calcium-phosphate metabolism, while the nitrogen, potassium, sodium, and chlorine were retained and excreted in an entirely normal way. Loss of calcium and phosphates in biliary fistula rickets, consequently, may possibly be only one part of a general metabolic disturbance and may lead, because it occurs in animals that are still growing, merely to what might be called a rachitic symptom complex. There is little doubt, however, that a lack of vitamin D has an important bearing upon the pathogenesis of this disease, which to a great extent proved to be curable by the administration of vitamin D. On the other hand, under the conditions described, a lack of this vitamin is not, most probably, the only cause of the entire condition.

It has been shown by Greaves and Schmidt (11) that bile is essential to the absorption of carotene. It might be found in further studies that vitamins other than A and D are involved, vitamins, for instance, that might not be absorbed or utilized in biliary fistula animals. Experiments are needed to clarify the rôles of the different causative factors, the elimination of which might finally lead to the experimental production of a purely rachitic state.

Metabolism studies of substances such as nitrogen, potassium, sodium, and chlorine, which are not subject to reexcretion through the intestinal wall—studies which led to entirely normal figures in rachitic infants as well as in rachitic rats—do not favor the so called absorption theory of Howland (8). This theory assumes that in rickets the small intestines fail to absorb calcium and phosphates normally. If it had been found that the absorption of nitrogen, potassium, sodium, and chlorine was interfered with in rickets, a failure in the absorption also of phosphates and calcium would, by analogy, become more probable. To assume, however, only an isolated failure to absorb phosphates and calcium, substances that are subject to reexcretion through the large intestine, seems rather arbitrary. Even though the discovery of the so called beryllium rickets by Kay and his coworkers (12) has estab-

lished that a failure to absorb phosphates may be one of different possible causes for the production of rickets in rats, there is thus far no conclusive evidence that this should also be the mechanism in infantile rickets³ (9).

SUMMARY

1. Rickets developed in three puppies deprived of vitamin D and sunlight since birth, in which, at the age of 6 to 7 weeks gall bladder fistula was established. The results of studies of their bones and of the calcium and phosphate metabolism have previously been published (2). Studies on the nitrogen, potassium, sodium, and chlorine metabolism, here presented, reveal that the metabolism was greatly interfered with as compared with that in three controls without gall bladder fistula rickets. This interference, together with an inhibited gain in weight, demonstrates that the pathogenesis of biliary fistula rickets in puppies has to be considered distinctly different from infantile rickets as well as from rickets produced in rats.

2. The nitrogen, potassium, sodium, and chlorine metabolism was also studied in three rachitic and four healthy infants, and in eleven rachitic and six control rats. The balance of these substances, as well as their distribution in stool and urine, proved to be the same whether or not rickets was present.

3. The pathogenesis of biliary fistula rickets is discussed on the basis of these studies. The assumption has been made that deficiency in other vitamins than vitamin D might have a bearing upon the development of this disease. Further study with a view to possible elimination of these unspecific factors might lead to the experimental production by gall bladder fistula of a purely rachitic state.

4. The normal balance and distribution of nitrogen, potassium, sodium, and chlorine in the stool and urine of rachitic infants and rats are considered further evidence of the hypothetical nature of the so called absorption theory in infantile rickets.

³ It might be mentioned here that the rate of intestinal absorption of carotene in two rachitic infants, determined by means of a method recently described (13), was found to be entirely normal in both infants: infant SL absorbed, on three different occasions, 78, 76, and 82 per cent of the amount of carotene in oil given by mouth, and infant BH, on two different occasions, 55 and 67 per cent.

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STUDIES ON INFLAMMATION

XII. MECHANISM OF INCREASED CAPILLARY PERMEABILITY. A CRITIQUE OF THE HISTAMINE HYPOTHESIS*†

By VALY MENKIN, M.D.

(From the Department of Pathology, The Harvard Medical School, Boston)

PLATE 34

(Received for publication, June 4, 1936)

Inflammatory changes are initiated with disturbances in local fluid exchange. There is an early vasodilatation accompanied by an increase in capillary pressure (1). This seems to be referable to a stimulation of the axone reflex. Many years ago Cohnheim pointed out that capillary permeability increased as a result of direct endothelial injury (2). The augmentation in seepage through the capillary wall may be detected as early as 2 minutes after the introduction of an irritant (3). The studies of Ricker and Regendanz, of Florey, and of Landis indicate that it is primarily injury and not mere dilatation of the capillary wall which increases its permeability (4-6). The consequent outpouring of plasma proteins reduces the osmotic pressure of the plasma colloids in the blood and thus enhances the effectiveness of the hydrostatic pressure in promoting the passage of fluid into the extracapillary spaces. The studies of Hudack and McMaster (7, 8) indicate that in the initial phase of the inflammatory reaction there seems likewise to be an increase in the permeability of the afferent lymphatics with probably an accompanying increase in the lymph flow. As the inflammatory reaction develops and if it progresses in intensity the lymphatic drainage becomes impeded by the presence of

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thrombi in the lymphatic channels and coagulated plasma in the inflamed area (9-11).

In earlier studies we pointed out that the initial increase in capillary permeability is readily demonstrable by the accumulation from the circulating blood of various substances at the site of inflammation. These include a dye, graphite material, and bacteria. With the types of irritants employed quantitative measurements revealed that the capillary permeability was increased twofold in acute inflammation (12-14).

The precise mechanism involved in the initial augmentation in capillary permeability in injury is obviously of considerable importance. Several years ago Lewis postulated the development of a type reaction primarily referable to a chemical H substance liberated from injured tissue. The H substance is presumably histamine or a substance having similar properties and therefore resembling it closely (15). According to Lewis and Grant the type reaction as elicited by the cutaneous injection of histamine manifests itself in three ways: (a) a local vasodilatation of capillaries, venules, and arterioles by direct action; (b) a widespread dilatation of outlying arterioles resulting from a local reflex; and (c) an increase in the permeability of the minute vessels by direct action (16). This type reaction leads to local edema of the skin. They conclude that:

"The vascular reactions of the skin in urticaria facticia in response to single strokes, and incidentally of normal skins to repeated strokes, are closely compared. The reactions are in general and in detail identical with those of the type reaction. From this comparison, and from further evidence of a more direct kind, it seems clear that the reactions of the skin to stroking result from the liberation of a chemical substance in the skin, this substance having a histamine-like action on the blood vessels and nerves. Less extensive comparisons of the skin's response to scratches and pin pricks, and to excessive heat, indicate that these responses are of a similar or identical kind and that these are determined by a chemical stimulus."

The conclusions of Lewis and Grant appear to be based largely on an analogy of the type reaction obtained by histamine with that of a variety of other injurious agents. When more direct tests were performed on the effect of the skin wheal fluid on the contraction of the guinea pig uterus, they were unable to obtain any evidence that histamine was liberated in larger quantities from injured tissue than was found in normal plasma. Nevertheless Lewis (15) concludes later that:

"It is difficult to refrain from stating without reserve the simple conclusion that the vasodilator substance considered and the H-substance are one and the same, and that this substance is histamine, free or held in loose combination." Further on he continues: "Nevertheless there are instances of response to skin injury in which disintegration products cannot be neglected entirely as a possible factor. It is desirable that they should still be remembered and further considered, and for this reason I shall continue to refer to H-substance in preference to histamine."

Krogh (17) accepts unreservedly the view first foreshadowed by the work of Ebbecke (18) and later chiefly sponsored by Lewis that under certain stimuli the tissue cells will liberate substances having a dilator effect on capillaries; but at the same time he finds it impossible to assume that in all cases the action is referable to a single chemical H substance. For this reason Krogh postulates the possibility of two effective substances liberated from injured cells: a diffusible factor closely related to histamine, if not histamine itself; and an H colloid substance which is probably less diffusible. Rous and Gilding (19) raise considerable doubt concerning the validity of a hypothesis which refers all local vasodilatation to the action of a single chemical substance liberated within tissues. They clearly demonstrated that the vascular contraction in Bier's spots prevailed over the local reddening induced by mechanical injury, whereas it was without effect upon the local vasodilatation induced by cutaneous injection of histamine. More recently Goldschmidt and McGlone (20) have studied the failure of a reactive hyperemia occurring with arrested circulation in an oxygen atmosphere. They have compared their findings with the histamine reaction in a similar oxygenated and ischemic environment. Their observations on the human forearm seem incompatible with the view that the vasodilatation responsible for reactive hyperemia is due to an H substance identical with histamine.

The present study was undertaken in order to determine whether one or more substances could be obtained from inflammatory exudates which, when introduced into normal cutaneous tissue, would induce local vasodilatation and an increase in the permeability of the capillary wall. Furthermore the properties of the active fractions which have been obtained from inflammatory exudates have been compared with histamine in an endeavor to test Lewis' hypothesis. In brief, the experiments about to be described indicate that a diffusible crystalline-like material capable of increasing capillary permeability is

present in inflammatory exudates. By appropriate tests this active principle has been shown to lack the properties characteristic of histamine, thus apparently ruling out the latter as of any primary significance in inflammation. Studies reported in a preliminary communication suggest that the twofold increase in potassium content found in exudates, as compared to blood serum, even as early as the first few hours of the inflammatory reaction may be connected in some way with the active factor (21).¹ In addition it was pointed out that organic compounds, other than histamine, including various products of proteolytic breakdown, such as amino acids, usually found increased in concentration in an exudate, likewise seem to have some effect in augmenting the permeability of capillaries during the course of the inflammatory process. The present report, however, will be confined to a description of the procedure adopted in isolating the active principle from an inflammatory exudate and to compare it in its properties to histamine. Studies on its chemical identification will form the subject of separate future communications.

EXPERIMENTAL

Inflammatory exudates, obtained by several different techniques, were studied. A convenient method of obtaining an abundance of exudative material is through the intrapleural injection of about 2 cc. of turpentine in dogs (22). Several experiments were also performed with exudates produced by the injection of minute amounts of croton oil in olive oil into the thoracic cavity of rabbits. In addition small quantities of exudate were obtained in the rabbit from cutaneous areas of inflammation induced by *Staphylococcus aureus*. Finally, in a few instances, to rule out the possibility of an admixture of an injected irritant with the exudate, sufficient edematous material was recovered by immersion, under ether anesthesia, of a rabbit's fore limb into almost boiling water for 1½ minutes.

The Presence in Inflammatory Exudates of a Substance Inducing Increased Capillary Permeability

As a rule the exudates were obtained from the thoracic cavity of dogs. The duration of inflammation ranged from 1 to several days. 0.2 cc. of the centrifugized

¹ The average potassium content of 14 blood serum determinations on dogs was 18.2 mg. per 100 cc. in comparison with an average level of 36 mg. per 100 cc. in a similar number of determinations on exudates. Osterhout pointed out that with injury to a cell there is a fall in potential difference and a consequent establishment of a current with outward migration of potassium ions (25). The possible implications of these findings to problems of permeability in inflammation are obvious and are accordingly being studied further.

cell-free exudate was injected into the normal skin of a rabbit.² This was immediately followed by the injection of 10 or 15 cc. of 1 per cent trypan blue in saline into the ear vein of the same animal. In the great majority of cases the treated cutaneous areas stained intensely and practically homogeneously with the dye. The accumulation of trypan blue occurred as early as 1 to 3 minutes after the intracutaneous inoculation of the cell-free exudate. The intensity of the staining in the injected area increased with time, reaching a maximum in about 10 to 15 minutes (see areas 4 and 4 a, Fig. 1). The cell-free exudate, when diluted 1 part in 10 with serum or water, still manifested an effect on the permeability of normal capillaries. In this dilution, however, the staining was relegated to the periphery of the treated skin areas.

Careful comparative studies have shown that the presence of small amounts of the active factor in a diluted exudate or as found in undiluted blood serum will invariably produce staining only of the outlying portion of the treated skin area. This difference in staining pattern is a convenient gauge of the concentration of active substance affecting capillary permeability in a given amount of body fluid material.

To rule out the effect of turpentine *per se*, this substance was suspended in a volume of water corresponding to the amount of exudate likely to be recovered from an intrapleural injection of 2 cc. of this irritant. The intracutaneous injection of this turpentine emulsion failed to produce, in the time required for the usual experiment, an accumulation of dye from the circulating blood stream. Furthermore an exudate obtained from inflamed tissues induced by a burn responded as actively as did the exudates produced by turpentine. When the edematous fluid of a 1 day old inflammatory area caused by *Staphylococcus aureus* was inoculated into normal skin tissue and a dye was injected intravenously, the cutaneous area was diffusely stained within several minutes. The active factor was evidently not the microorganism *per se*, for in an equal interval of time a heavy broth

² The same experiment was repeated by injection of the cell-free exudate, obtained from a dog's pleural cavity, into the skin of a normal dog. In a few instances a pleural exudate removed from a rabbit was injected into its own normal skin; and this was followed by intravenous injection of the dye. These additional controlling experiments were done in order to rule out any question of protein specificity on capillaries by using body fluids of one animal and injecting this material into another animal.

suspension of *Staphylococcus aureus* failed to cause any accumulation of dye when injected intracutaneously into the same rabbit.

When the serum of a normal dog or rabbit is treated in the same manner as an exudate, the dye, in the interval of time studied, usually either fails to permeate from the circulating blood, or only a relatively small amount passes through, staining exclusively the periphery of the injected cutaneous area. For the reason stated above, this seems to indicate that the active factor inducing increased filtration through the endothelial wall exists in definitely lower concentrations in blood serum than in exudates. The foregoing observations suggest that a substance other than the inflammatory irritant which is capable of inducing almost immediate increased capillary filtration is present in an inflammatory exudate.

Studies on the Concentration and Isolation from Exudates of a Factor Effective in Increasing Capillary Permeability

Experiments were set up in an endeavor to determine the nature of the active factor liberated in injured tissue which is capable of inducing an increase in capillary permeability. Various obvious questions presented themselves from the start. Was one dealing with the protein fraction of the exudate, and if so, precisely which of the recognized plasma proteins were involved? On the other hand, was it not possible that one was concerned with a diffusible crystalline substance, such as histamine, or one of its derivatives, as implied in the studies of Lewis? Or perhaps an inorganic ion was the active factor. Then finally might not the increased capillary permeability be the resultant of several different but reinforcing substances some of which might have been introduced with the irritant, and others liberated as the degree of local injury progressed? The first of these questions was studied in the following manner.

Varying amounts of exudate, obtained as described above, were treated with an equal volume of saturated ammonium sulfate. A heavy precipitate formed. This readily redissolved in distilled water, saline (0.16 M NaCl), or in a phosphate buffer mixture at pH 7.35. As a rule the resulting solution produced a prompt increase in the filtration of the normal capillary as indicated by the immediate accumulation of trypan blue from the circulating blood into the treated cutaneous area of a rabbit (see area 2, Fig. 1). The reaction was not referable to distilled water which invariably induced vascular contraction, as manifested by conspicuous local blanching. In such an area the dye failed to accumulate for a consider-

able interval of time. The buffer mixture or saline *per se* likewise induced no local staining reaction. Ammonium sulfate, as well as sodium sulfate, produced an entirely different picture when injected into the dermis. The appearance in the case of these two salts was identical, suggesting that the type reaction was referable to the $\text{SO}_4^{=}$ in both cases, rather than to the cations. This response was characterized by an inner colorless zone surrounded by an area of pronounced congestion peripheral to which a narrow zone of blue stood out conspicuously (see areas 7 and 8, Fig. 1). This pattern obviously differed entirely from the local reaction observed either when the untreated cell-free exudate or the saturated ammonium sulfate precipitated fraction were compared (areas 4, 4 a, and 2, Fig. 1). In a number of instances, though not invariably, the local effect on the capillary obtained with the ammonium sulfate fraction appeared to be more intense than when the untreated cell-free exudate was employed. Results of the same type were obtained when a precipitate was produced by the interaction of 20 per cent sodium sulfate with the cell-free exudate (see area 1, Fig. 1).

When the globulin fraction of the exudate was precipitated out by treatment with one-half or one-third saturated ammonium sulfate and the precipitate was redissolved in a buffer mixture, the solution, as a rule, produced no effect on the normal capillary wall. The suspended precipitate obtained either by treating the exudate with 95 per cent alcohol or 5 to 20 per cent trichloroacetic acid likewise proved to be inactive. As compared to exudate, blood serum showed either no effect or at most a diminished activity on the endothelial wall. However when blood serum was treated with saturated ammonium sulfate a precipitate formed which, dissolved in a buffer mixture, often enhanced the filtration of dye into the treated skin area. This would suggest that the saturated ammonium sulfate precipitated fraction represents perhaps in a concentrated form a factor found in abundance in inflammatory exudates and in smaller quantity in blood serum.

For the convenience of the reader a type protocol of the experiment illustrated on Fig. 1 is presented (Table I). The data are self explanatory. The number of plus signs refers to the intensity of staining by the dye in the various local skin areas, and the time intervals are stated indicating the rapidity of passage of dye into the extracapillary spaces.

At this stage of the investigation the observations suggested that the active factor concerned might possibly be the albumin fraction of the exudate or a substance carried down with this protein upon treatment with certain precipitants. For this reason the analysis was pursued by adopting the scheme shown in Table II.

The precipitate obtained by treating the cell-free exudate with saturated ammonium sulfate was dialyzed for several hours in a cellophane bag against distilled water. The dialyzed protein material remaining in the cellophane bag was found

to be inactive when injected into the normal skin of a rabbit. Trypan blue failed to permeate into the site of skin inoculation which assumed an appearance of local blanching (area 3, Fig. 2). This dialyzed fraction was acid to phenol red, but when it was rendered alkaline by dissolving the protein in a buffer mixture of pH

TABLE I

Protocol of Dog 7-0. Experiment. Exudate and its fractions. Nov. 15, 1935.

Cutaneous area No., inoculated with	Time of inoculation <i>hrs.:min.:sec.</i>	10 cc. 1 per cent Trypan blue in saline intravenously at 5:32:5		
		Presence of dye in inoculated area at		
		5:35	5:40	5:45
(1) Na ₂ SO ₄ (20%) treated fraction dissolved in buffer pH 7.35	5 : 27	0	+	++
(2) (NH ₄) ₂ SO ₄ (satura- ted) fraction dis- solved in buffer pH 7.35	5 : 27 : 5	(?) Slight trace	+	++
(3) Trichloroacetic frac- tion suspended in buffer pH 7.35	5 : 28	0 (blanching)	0 (blanching)	0 (blanching)
(4) Untreated cell-free exudate	5 : 28 : 5	+	+++	++++
(4 a) Same as (4)	5 : 28 : 7	Slight trace	++	++++
(5) Evaporated (NH ₄) ₂ SO ₄ frac- tion and redis- solved in buffer pH 7.35	5 : 29 : 0	+	++	+++
(6) Buffer pH 7.35	5 : 29 : 5	0	0	0
(7) 20% Na ₂ SO ₄	5 : 30	Center 0. Pe- ripheral zone of redness	+ Peripheral to inner zone of redness	Same as at 5 : 40
(8) Saturated (NH ₄) ₂ SO ₄	5 : 32	Same as (7)	Same as (7)	Same as (7)

7.43, it likewise failed to enhance seepage of dye from the circulating blood (area 3 a, Fig. 2).

These observations indicated that the albumin fraction of the inflammatory exudate was evidently not the factor responsible for increased capillary permeability in inflammation. Further substantia-

cell-free exudate
Dog 91 exp 4/16 g p.g. intestine

histamine
5 mg / cc
(Dog 91 exp of 2/4/36)

TEXT-FIG. 1. 1a represents the effect on the strip of intestine of the cell-free exudate. 1b represents the effect of histamine on such a segment.

↑
cell-free exudate dog 92 (2/7/36)
diluted 1:4 in rabbit serum
intestine

in the strip of intestine of an inflammatory exudate

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tion of this point was obtained when crystallized serum albumin or crystalline egg albumin³ was introduced into the dermis of a normal rabbit. Trypan blue failed to accumulate in cutaneous areas treated with these pure proteins. The analysis was consequently directed towards testing for the possible presence of diffusible active substances in the dialysate.

The dialysate, whether untreated, or freed of its $\text{SO}_4^{=}$ ions by repeated treatment with 10 per cent barium chloride, was evaporated to dryness either on an ordinary water bath or, as in the preliminary experiments, in a vacuum oven under reduced temperature. The material obtained by this procedure appeared as a mass of crystals, heterogeneous in respect to size and shape. Its reaction was, as a rule, alkaline to phenol red. The Fehling test was positive, indicating the presence of reducing substances in the dialysate. The biuret and nitric acid tests for proteins were negative.

It is quite obvious that this crystalline material was in no sense purified and therefore doubtless represented a mixture of several diffusible substances. The crystalline water soluble fraction obtained from the evaporated dialysate showed definite activity in regard to the augmentation of capillary filtration (Table II). Trypan blue from the circulation accumulated throughout the local skin area, staining it relatively homogeneously. This occurred irrespective of whether the crystalline material was dissolved in water or whether the dialysate was simply concentrated to a very small volume by evaporation (see Fig. 2, areas 1 and 2 a).

The active permeability factor, as found in the untreated cell-free exudate, or as obtained in its dialysate, retains its potency when the material is kept on ice. In the types of acute inflammation studied (23) the activity is not altered by changes in the pH of the exudate, ranging from 7.4 to 6.5. The active substance is essentially thermostable. It may still display definite activity after being brought to the boiling point. Freezing it down to -20° does not seem to alter its potent effect on the capillary wall. Dialysis of the untreated cell-free exudate without preliminary treatment with saturated ammonium sulfate produced practically the same type of result, except that here the material that remains in the cellophane bag sometimes retained a slight degree of activity in affecting the capillaries. It induced con-

³ Obtained through the courtesy of Dr. E. J. Cohn.

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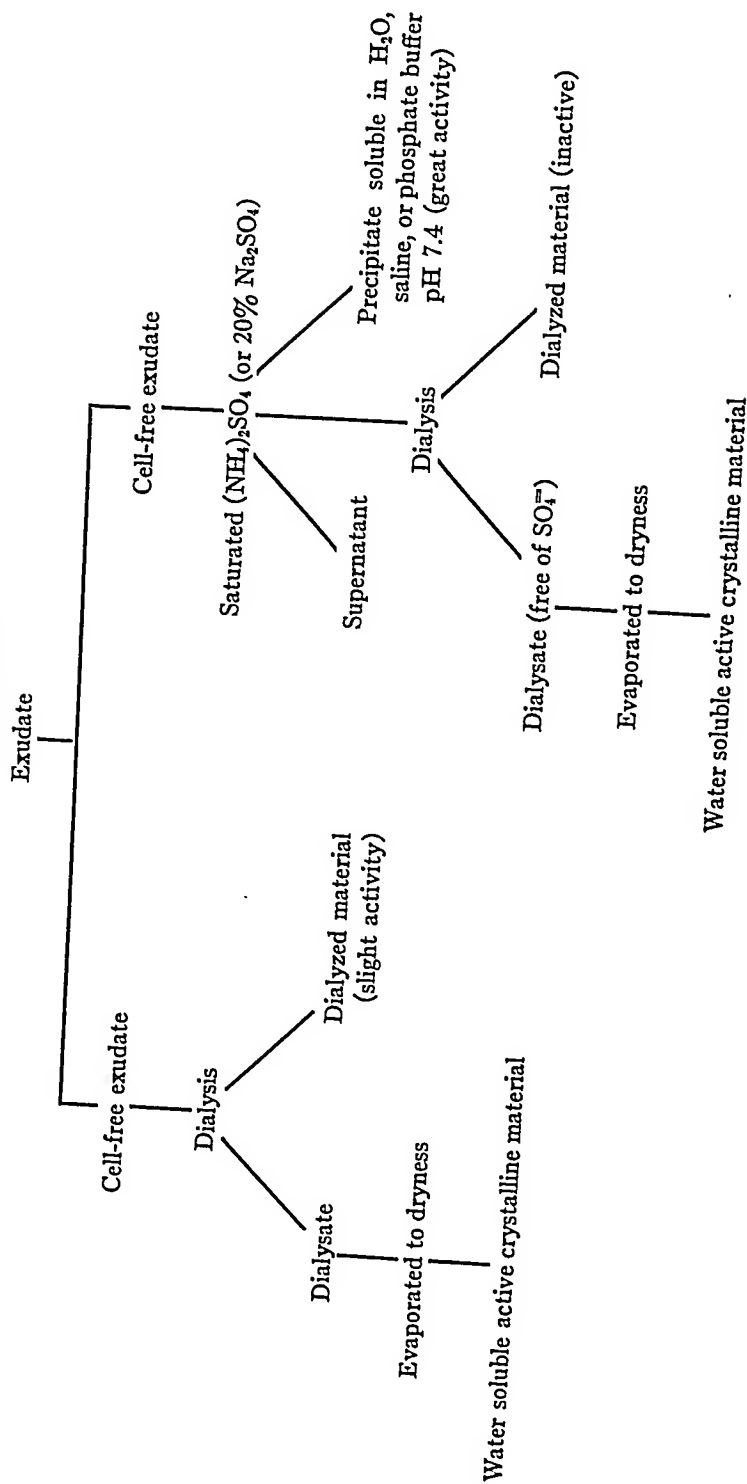
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TABLE II
Scheme of Extraction



centration of the dye, but only at the periphery of the treated skin area (Table II).

To conclude, the results obtained from various types of inflammatory exudates suggest the presence of some agent which almost immediately augments filtration of a dye through the normal capillary wall. Previous work (13, 14) indicated that this increased filtration was primarily referable to changes in the permeability of the capillary endothelium. Furthermore the active factor seems in large part to be of crystalloid dimensions and therefore diffuses fairly readily through a cellophane membrane. Finally the active diffusible factor appears to exist also in blood, although biological tests indicate that it is present there in definitely smaller quantities than in an inflammatory exudate.

Is Histamine the Active Factor Found in Inflammatory Exudates?

In the introductory section of this paper mention was made of Lewis' hypothesis to the effect that the mechanism of increased capillary permeability in injury was referable to the liberation of a single substance, presumably histamine, or at least a closely related compound, the so called H substance. In an endeavor to confirm this view the properties of histamine were compared with those of the active factor found in inflammatory exudates.

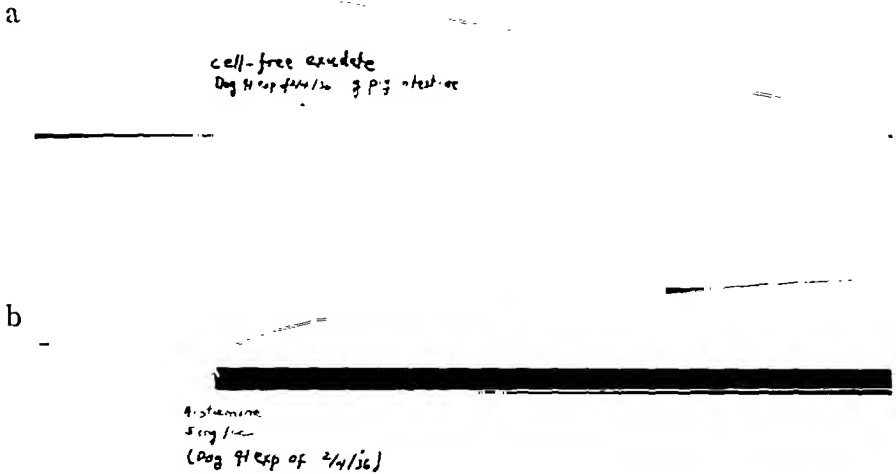
Histamine (ergamine acid phosphate) was dissolved in either water, serum, or saline in varying amounts ranging from 0.1 to 10 mg. In each case the given quantity of histamine was taken up in 0.2 cc. of fluid and injected intracutaneously into the abdomen of rabbits. Trypan blue was then introduced into the ear vein. The dye either failed to enter the histamine-treated area or else, in some cases, it diffused in a widespread flare-like formation at the periphery of the site of inoculation, with a conspicuous area of local blanching appearing in the center (area 7, Fig. 2). This outlying zone of staining was more likely to occur with the high concentrations of histamine.

The obdurate reaction to histamine on the part of the capillaries in the rabbit had been pointed out recently by Morgan (24). In the dog, however, trypan blue accumulated readily in a histamine-treated area, but here again the dye displayed the characteristic flare-like pattern at the peripheral portion of the local area of inoculation, the center of which remained blanched. This occurred even when 2.75

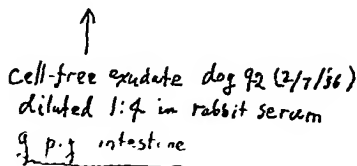
mg. of dissolved histamine had been introduced into the skin of the dog. In contrast to the staining pattern obtained with histamine, it has already been pointed out that the active factor found in an exudate or recovered from its dialysate produces a homogeneous or practically uniform effect on the capillaries of the injected skin area. This results in a relatively even staining by the dye as it concentrates from the blood stream into the extracapillary spaces of the inoculated area (see areas 4 and 4 a, Fig. 1; areas 1 and 2 a, and 6, Fig. 2). As previously stated, diluting the active factor found in an exudate results merely in a peripheral localization of the dye when the samples are tested on the normal skin. This is likewise true of undiluted blood serum. In view of the dilution experiments with the cell-free exudate, it has already been suggested that the active capillary factor is probably found in definitely smaller quantities in blood than in inflammatory exudates. Nevertheless, both with diluted exudate or undiluted serum, the local staining of the site of cutaneous inoculation never spreads in the outlying fashion characteristic of histamine. Furthermore the staining pattern of histamine occurs when as much as 10 mg. of this substance is introduced into a local skin area. It is doubtful whether such a high concentration of this substance would be exceeded in 0.2 cc. of a cell-free exudate. It seems therefore unlikely that the distinction between the tissue stained patterns of undiluted exudates and of histamine is in any way referable to a superabundance of histamine in inflammatory fluids.⁴

⁴ An additional corroborative evidence of the antagonistic action of histamine to the active principle was obtained by the addition of 1 mg. of histamine to 0.2 cc. of an otherwise potent exudate. Areas on the dermis of the abdomen of rabbits treated with such preparations were found to be refractory in regard to seepage of dye from the circulation.

The studies of G. S. Barsoum and J. H. Gaddum recently pointed out that if the addition of histamine to an unknown extract induces the disappearance of the original contractile effect of the extract on an isolated intestinal segment this may serve as indication that the effect of the extract is due to histamine (*J. Physiol.*, 1935, 85, 1). For this reason an experiment was set up in which, after treating the intestinal guinea pig segment with the active dialysate of an exudate, a solution of histamine was added to the same bath. The usual depressing effect of the exudate or of its dialysate on the tonus of the intestine was immediately replaced by powerful tonic contractions, showing thus that there was no evidence of any refractory state to histamine induced by preliminary treatment with the inflammatory exudate.



TEXT-FIG. 1. 1a represents the effect on the strip of intestine of the cell-free exudate. 1b represents the effect of histamine on such a segment.



TEXT-FIG. 2. The effect on the strip of intestine of an inflammatory exudate diluted 1:4 in rabbit serum.

TEXT-FIG. 3. The addition to the isolated strip of intestine of an aqueous solution of the active crystalline material recovered from the dialysate of an inflammatory exudate. This was followed by the addition of histamine, as indicated. Compare on the one hand the lack of response induced by the active crystalline material with the pronounced contractile reaction obtained by histamine.

In order, however, to establish any further differences in properties between histamine and the active capillary factor found in an inflammatory exudate, the following tests were made. Histamine is known to increase the tonus of the guinea pig intestine or of the virgin uterus. This effect is readily elicited and recorded on a revolving kymograph as a contraction of the isolated segment. Although several experiments were tried with the uterus most of the observations were made by tests on the isolated intestinal loop, comparing thus the effect of histamine, cell-free exudate, and the crystalline material obtained from the dialysis of the exudate. When histamine in the various concentrations employed for the skin tests is added drop by drop to a suspended loop of intestine, a prompt contraction of the segment ensues (Text-fig. 1 b). When, on the other hand, the cell-free exudate is added to a fresh loop the intestinal segment definitely relaxes, as indicated by a decrease in tonus and a definite drop of the recording pointer (Text-fig. 1 a). Precisely the same decrease in tonus is obtained if the exudate is diluted 1 part in 4 in rabbit serum (Text-fig. 2). In this connection it is to be recalled that dilution of the exudate produces merely peripheral localization of dye in the treated skin area. Nevertheless the depression in the tonus of the intestine persists in contrast to the contractile effect of histamine. When the crystalline material obtained by evaporating the dialysate of an exudate is tested on the loop of intestine, it likewise fails to induce contraction, whereas the subsequent addition of histamine may give rise to a powerful contraction (Text-fig. 3). Undiluted blood serum, which in itself may occasion merely peripheral staining of a treated skin area, fails, like the exudate, to produce any contraction of the isolated intestinal strip.

In order to ascertain whether a human inflammatory exudate, in contrast to histamine, elicits the same reactions as the exudates of dogs or rabbits, identical tests were repeated on a sample of fluid removed from the chest of a patient with a hemolytic streptococcus infection.⁵ The prompt uniform staining reaction on the dermis of the abdomen of a rabbit and the response of the isolated guinea pig intestine were precisely the same as in the samples from experimental animals, differing thus entirely from the reactions obtained with histamine.

⁵ This exudate was obtained from the Beth Israel Hospital through the courtesy of Dr. H. L. Blumgart and Mrs. D. R. Gilligan.

The above observations indicate that the active factor recovered from an inflammatory exudate, which is capable of inducing increased capillary filtration primarily by injury to the endothelial wall, does not seem to be histamine nor is it the H substance in so far as its properties are supposed to resemble closely those of histamine. This is revealed by comparing histamine and the active factor found in exudates in regard to their local effects on the capillary wall, as evidenced by the differential staining patterns produced and also by the opposite types of response which they induce on the isolated strip of intestine.

SUMMARY AND CONCLUSIONS

Various types of inflammatory exudates have been obtained either by the introduction into normal tissues of a chemical irritant, or by a burn, or by bacteria in either dogs or rabbits. A study has also been made on an exudate of human origin.

These exudates have all been found to contain a factor which induces prompt increase in the permeability of normal skin capillaries, demonstrable by the almost immediate accumulation from the circulation of trypan blue into areas of skin injected with the cell-free exudate.

The active factor may be carried down with the precipitate resulting from the interaction of the exudate with either saturated ammonium sulfate or 20 per cent sodium sulfate.

The active factor passes through a dialyzing membrane. It can be recovered from the dialysate as a protein-free crystalline material.

The active factor manifests no property in common with histamine or presumably with the hypothetical H substance assumed to be closely related to histamine.

This is indicated by the following considerations: (a) difference between the tissue staining pattern of the exudate or of its active fraction and that of histamine; (b) opposite effects by histamine and the active factor found in exudates on the tonicity of the isolated strip of guinea pig intestine.

The observations presented in this report do not substantiate Lewis' hypothesis of histamine or of its closely related H substance as the primary cause of increased capillary permeability in inflammation.

The present studies are being continued in an endeavor to free of its impurities and to identify the active crystalline-like material iso-

lated from an inflammatory exudate. The details of this investigation will form the subject of a separate future communication.

We greatly appreciate the technical assistance of Mr. M. Kadish during the course of this investigation.

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EXPLANATION OF PLATE 34

FIG. 1. The dermis of the abdomen of a rabbit treated with an inflammatory exudate and some of its fractions. The skin inoculations were followed by the intravenous injection of 10 cc. of 1 per cent trypan blue in saline. The effect on the permeability of the capillary wall is determined by the extent of dye accumulation in the various skin areas treated. For an explanation of the material inoculated in each area the reader is referred to Table I in the text.

FIG. 2. The dermis of the abdomen of a rabbit treated with various fractions of an inflammatory exudate and with histamine. The skin inoculations were followed by the immediate intravenous injection of 1 per cent trypan blue. The effect on capillary filtration is indicated by the extent of the local staining reactions induced by the respective fractions. These were as follows:

No. 1. Cell-free exudate treated with saturated $(\text{NH}_4)_2\text{SO}_4$ and dialyzed. The dialysate was concentrated to about 1/17th of its original volume and inoculated in the skin. Note the considerable accumulation of the dye in the area.

No. 2 a. The dialysate in area 1 was treated with 10 per cent BaCl_2 to precipitate out the $\text{SO}_4^{=}$ ions. The supernatant fluid was diluted with an equal volume of phosphate buffer mixture (pH 7.43) and inoculated intracutaneously. The accumulation of dye is a conspicuous feature.

No. 3. The protein material of the exudate remaining in the cellophane bag after dialysis. The dialysate of this sample was injected after concentration into area 1. Note the inactivity of the protein fractions of the exudate.

No. 3 a. The same as area 3 with the exception that the protein material was rendered alkaline by diluting one part of it with an equal volume of phosphate buffer (pH 7.43).

No. 5. Some material flocculated and remained behind in the cellophane bag after dialysis of the exudate. This precipitate probably represents the euglobulin fraction of the exudate. Its inactivity is evident.

No. 6. The cell-free exudate treated with saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in a phosphate buffer mixture (pH 7.43). The effect is evident, indicating that the active material was carried down with the precipitate.

No. 7. The injection of 0.55 mg. of histamine dissolved in distilled water failed to cause the accumulation of trypan blue in the treated skin area. Compare this effect with that in area 1.

No. 8. Phosphate buffer mixture (pH 7.43).

No. 9. Distilled water.

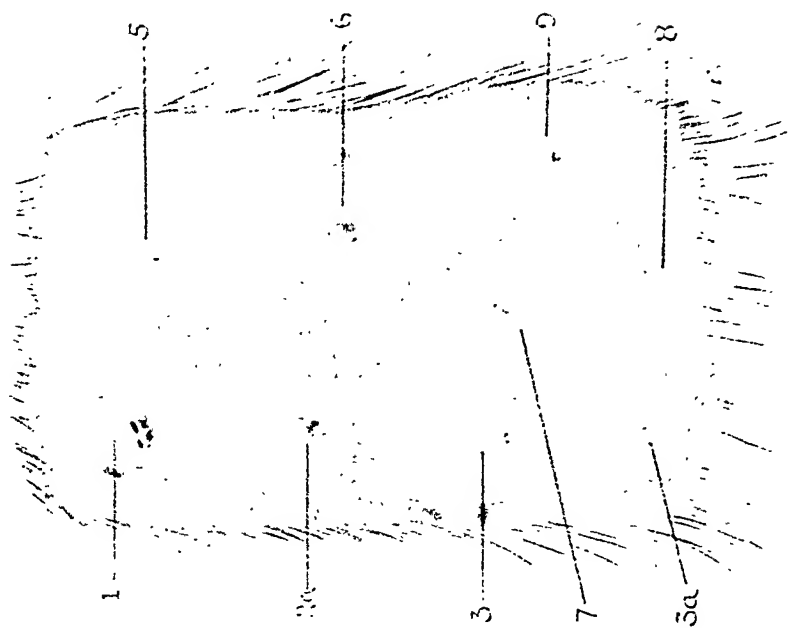


FIG. 2

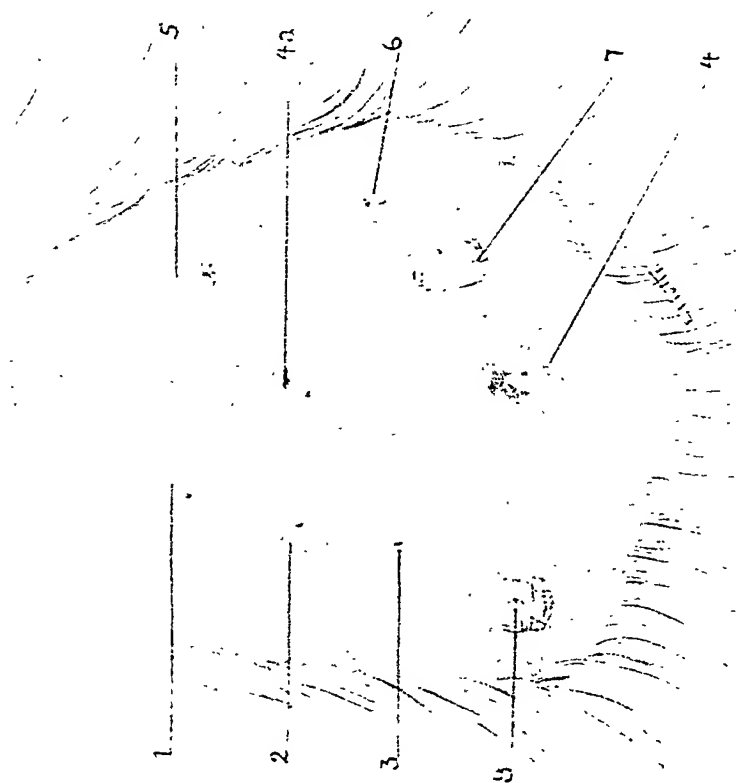


FIG. 1

A HIGH SPEED VACUUM CENTRIFUGE SUITABLE FOR THE STUDY OF FILTERABLE VIRUSES

By JOHANNES H. BAUER, M.D., AND EDWARD G. PICKELS, Ph.D.

*(From the Laboratories of the International Health Division,
The Rockefeller Foundation, New York)*

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The study of filterable disease-producing agents has been much handicapped by the lack of suitable apparatus. The microscope, the ordinary laboratory centrifuge, and culture medium, all of which are invaluable in the study of disease-producing bacteria, are of no assistance in the study of viruses. Viruses associated with disease in animal and man are also always associated with a large amount of extraneous protein matter originating from the host, and no satisfactory method has yet been developed by which these pathogenic agents can be induced into a pure and concentrated state so that their specific antigenic properties and physicochemical characteristics can be properly investigated. Recently Stanley (1) reported success in the preparation of the virus of tobacco mosaic in crystalline form by the extraction and salting-out process, but as yet this method has not found wide application in the study of animal and human viruses. As is well known, most of the viruses pathogenic to animal and man are rather labile and become easily inactivated by the processes involved in chemical and physical investigations, and since the presence of a virus in a given substance can be demonstrated only by its specific action in a susceptible animal, these methods have proven of little value.

The work of Craigie (2) and Parker and Rivers (3) on the vaccinia virus has shown that a centrifuge by which the elementary bodies can be not only separated from the associated protein matter but washed and concentrated as well, can be an exceedingly useful tool in the study of this virus. Similar application of the centrifuge to a considerable number of other viruses has not yielded equally satisfactory results. Within recent years, through the work of Elford (4) and Bauer and

Hughes (5), a method for the preparation of finely graded collodion membranes of uniform porosity has become available. By the use of these membranes the particle size of a number of viruses has been measured by various investigators. The results of these investigations are summarized in Table I. A glance at this table reveals a wide variation in their size. Assuming arbitrarily that virus particles are more or less spherical bodies with a density equal to that of most proteins, a calculation from the familiar Stokes' law would indicate that for a given length of time a centrifugal force several hundred times greater would be required to sediment the virus of foot and mouth disease than was successfully applied to vaccinia virus by the workers mentioned above. This will explain, at least in part, why most of the centrifugation experiments with various viruses in the past have not been successful. It is true that there are a number of commercial centrifuges available which give fairly high rotational speeds although most of them have a relatively short effective radius. But as they are generally designed to rotate in air, their speed is necessarily limited by the air resistance. Moreover, in such centrifuges, with the increase of speed a correspondingly greater amount of frictional heat is developed in the rotating head. This heating is not only responsible for convection currents which interfere with effective separation, but the temperature frequently rises to such a critical degree that denaturation and inactivation of the material during centrifugation may result.

Recently we reported (28) the construction of an air-driven centrifuge built on the principles described by Beams, Weed, and Pickels (29). This centrifuge was primarily intended for the separation and concentration of the yellow fever virus which, as shown in Table I, is one of the smallest viruses and which is of particular interest to us. Good separation was obtained in this centrifuge with some of the larger viruses, such as that of vesicular stomatitis, but the results with the yellow fever virus were entirely negative. Because this centrifuge rotates in open air and has a large surface area exposed to air drag, its speed is necessarily limited; and although an air pressure as high as 225 pounds per square inch was applied, a speed of 16,000 revolutions per minute could not be exceeded. This corresponds to a centrifugal force at the top portion of the fluid of only about 13,500

times gravity. Obviously this speed was insufficient, since it was estimated that a centrifugal force of at least 30,000 times gravity must be exerted before a definite sedimentation of yellow fever virus could be expected within a reasonable period of time. It was obvious also that any type of centrifuge designed to give the desired centrifugal force must be protected from air friction. In the centrifuge described

TABLE I

Approximate Particle Size of Viruses as Determined by Filtration through Graded Collodion Membranes

Virus	Estimated particle size <i>millimicrons</i>	Authority
Vaccinia.....	125-175	Elford and Andrewes (6)
Canary pox.....	125-175	Burnet (7)
Lymphogranuloma inguinale.....	125-175	Broom and Findlay (8)
Rous sarcoma 1.....	100-150	Elford and Andrewes (9)
Ectromelia.....	100-150	Barnard and Elford (10)
Pseudorabies.....	100-150	Elford and Galloway (11)
Herpes.....	100-150	Elford, Perdrau, and Smith (12)
Borna disease.....	85-125	Elford, Galloway, and Barnard (13)
Influenza, swine and human.....	80-120	Elford, Andrewes, and Tang (14)
Vesicular stomatitis.....	70-100	Galloway and Elford (15); Bauer and Cox (16)
Fowl plague.....	60-90	Elford and Todd (17)
Rift Valley fever.....	23-35	Broom and Findlay (18)
Equine encephalomyelitis.....	20-30	Bauer, Cox, and Olitsky (19)
St. Louis encephalitis.....	20-30	Bauer, Fite, and Webster (20); Elford and Perdrau (21)
Yellow fever.....	17-25	Findlay and Broom (22); Bauer and Hughes (23)
Louping ill.....	15-20	Elford and Galloway (24)
Poliomyelitis.....	10-15	Theiler and Bauer (25); Elford, Galloway, and Perdrau (26)
Foot and mouth disease.....	8-12	Galloway and Elford (27)

below the difficulties were entirely overcome when the rotor was placed in very high vacuum, and the results obtained with this machine have been satisfactory.

Driving Mechanism.—The driving mechanism is a modification of those described by Beams and Pickels (30) and later by Biscoe, Pickels, and Wyckoff (31). It is shown in Figs. 1, 2, 3, 4, and 5. Figs. 1 and 2 show vertical cross-sectional

views of the essential parts. The horizontal cross-sectional view in Fig. 3 gives further details of the turbine and driving jets. Fig. 4 is an actual photograph of the turbine and air-bearing units, and Fig. 5 pictures the complete driving assembly.

The rotating members are the turbine (1, Figs. 1, 3, 4), the drive shaft (2, Figs. 1, 4), and the rotor (3, Figs. 1, 5). The turbine is made of light phosphor bronze and the angle of its cone-shaped base is 90° . It is hollowed out to reduce its

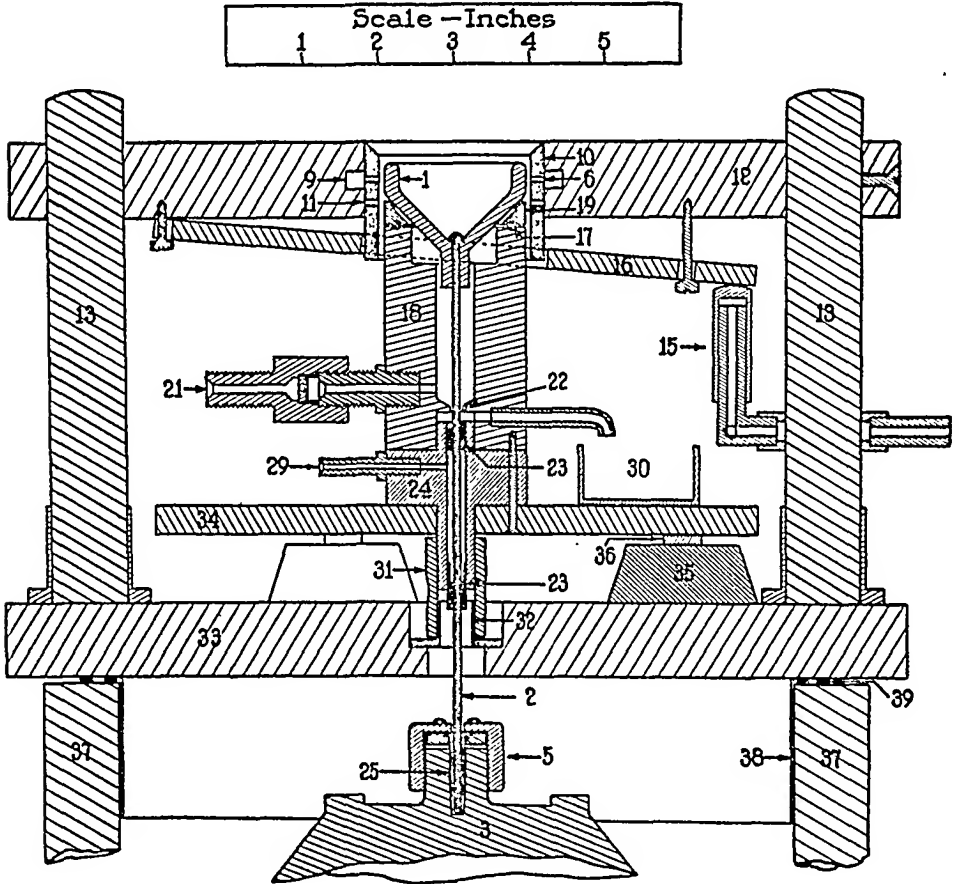


FIG. 1. Vertical cross-sectional view of the entire driving mechanism.

weight and is provided with nineteen flutings cut with a $\frac{3}{8}$ inch dovetail milling cutter to a depth (along the radius of the turbine) of about $\frac{3}{32}$ inch. The shaft is a straightened section of spring steel wire having a diameter of $\frac{1}{16}$ inch. Its upper end is snugly fitted and soldered into the turbine (care being taken not to overheat the wire and cause a loss of its temper) and its lower end is fastened to the rotor by a special chuck arrangement (5, Fig. 1). The driving power is supplied by air jets issuing from eight $\frac{1}{16}$ inch holes spaced and directed as illustrated (6, Figs. 1, 3).

The compressed air is conducted to these holes through the flexible pressure tubing (7, Fig. 5), the coupling (8, Fig. 3), and the distributor chamber (9, Figs. 1, 3). The brass sleeve (10, Figs. 1, 3) containing the forward jet holes is also fitted with eight reverse jet holes (11, Fig. 1), two of which are indicated in the background of Fig. 3 under 11. Each jet hole in both sets is directed along a line which is $\frac{1}{4}$ inch from the axis of rotation. The clearance between the sleeve and turbine is $\frac{1}{16}$ inch.

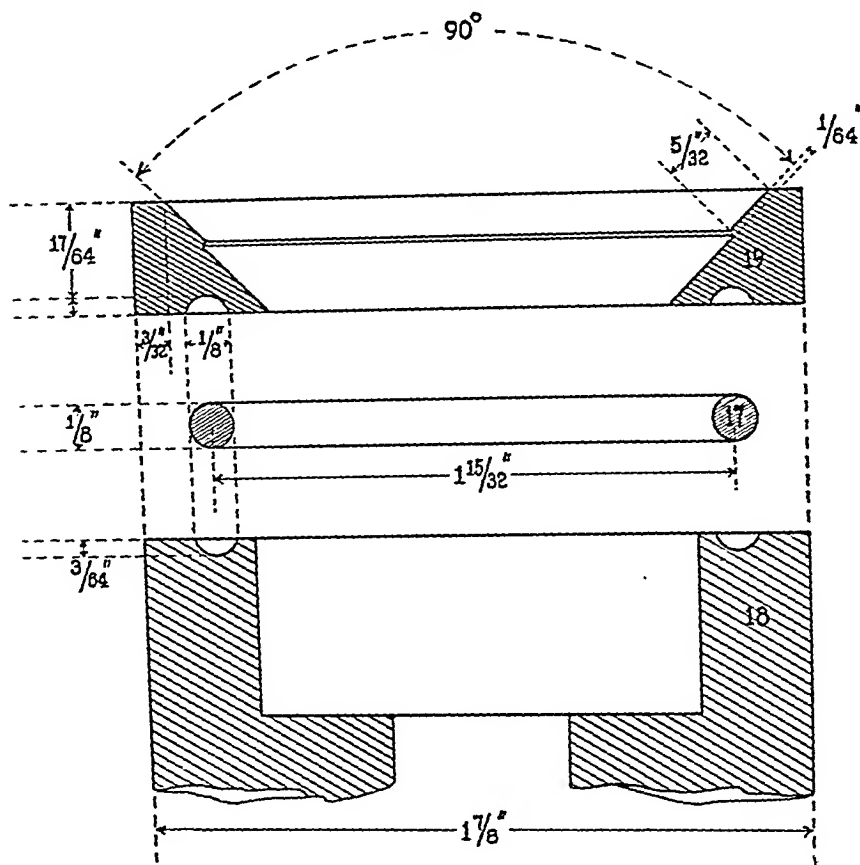


FIG. 2. Vertical cross-sectional view of the air-bearing units.

The sleeve can move up and down in the duralumin cross-arm (12, Figs. 1, 3, 5) which is supported by two steel rods (13, Figs. 1, 5). The reverse jets are put into operation by supplying compressed air through the rubber tubing (14, Fig. 5) to the air piston (15, Fig. 1). This raises the end of the brass plate (16, Figs. 1, 3) which in turn communicates the movement to the sleeve, 10, through two pins, 17, one of which is shown in Fig. 3. The other is directly opposite but has

been omitted to simplify the drawing. This action results in a shift of jet holes, 11, to a position adjoining the distributor chamber, 9, and a shift of the forward jet holes to a shut-off position above the chamber. action of gravity, the sleeve and plate drop back to their normal position the pressure is released.

The rotating elements are supported by the air-bearing arrangement as disassembled in Fig. 2, and as during operation in Fig. 1. A rubber ring (Figs. 1, 2, 4) is made by splicing together with rubber cement the ends of $\frac{1}{8}$ inch round rubber belting. This ring rests in a circular groove of support (18, Figs. 1, 2). The rubber supports and centers a bakelite ring (Figs. 1, 2, 4) as indicated. This ring is cut from ordinary black bakelite

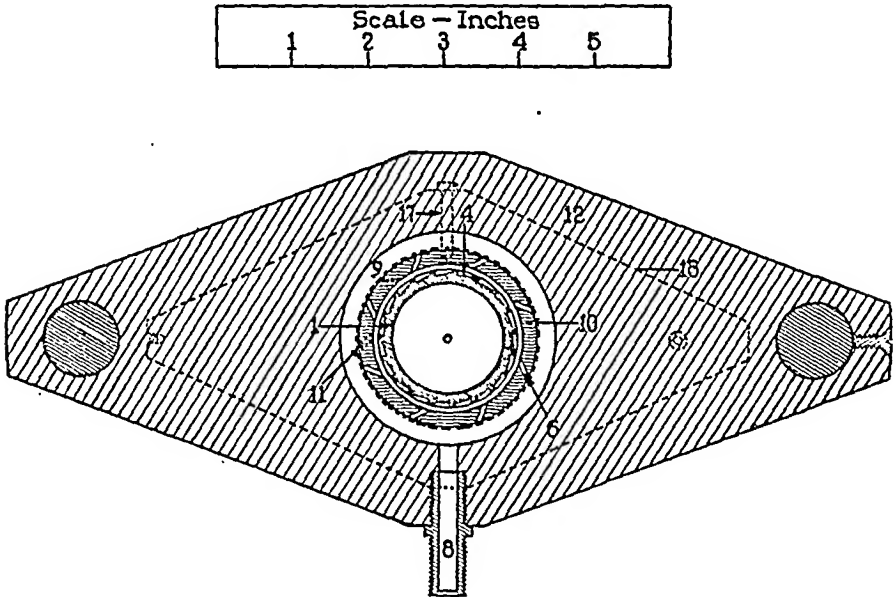


FIG. 3. Horizontal cross-sectional view of the turbine and driving jets.

board. The turbine actually rides on a thin film of air escaping between its conical surface and that of the bakelite ring. This air is supplied through the pressure tubing (20, Fig. 5) which is coupled to the fine wire screen filter (21, Fig. 1). The clearance (22, Fig. 1) about the wire just below the inlet hole from the coupling is made small enough to prevent any great loss of air, and yet sufficiently large to eliminate the possibility of the wire touching the edges of the hole. A $\frac{1}{8}$ inch hole was found to be satisfactory.

The shaft is guided by two phosphor bronze bushings (23, Fig. 1), each of which is prepared as follows: A bronze plug is machined approximately to size, and a central hole slightly smaller than the shaft is then drilled. The wire selected for the shaft is cut to a length several inches longer than actually needed for the apparatus. With the help of an emery wheel, one end is ground to the shape of a triangular, slightly tapered reamer. The hole of the plug is reamed to a snug fit

and the bushing then fitted on an arbor and turned to the correct size for forcing into the brass gland (24, Fig. 1). The same method of reaming and finishing on an arbor is used in fitting the shaft into the turbine and into the chuck (25,

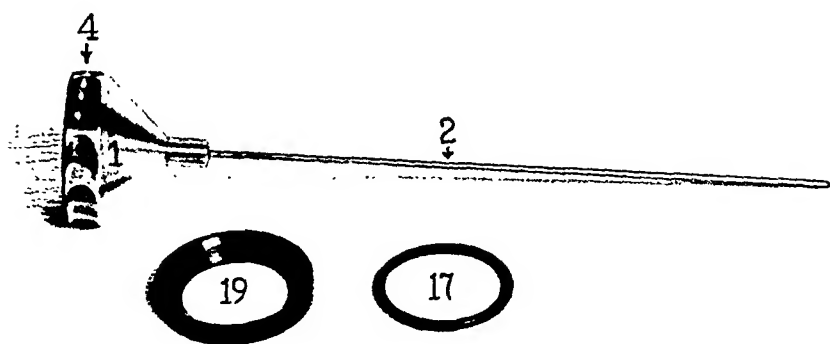


FIG. 4. Turbine and air-bearing units shown separately.

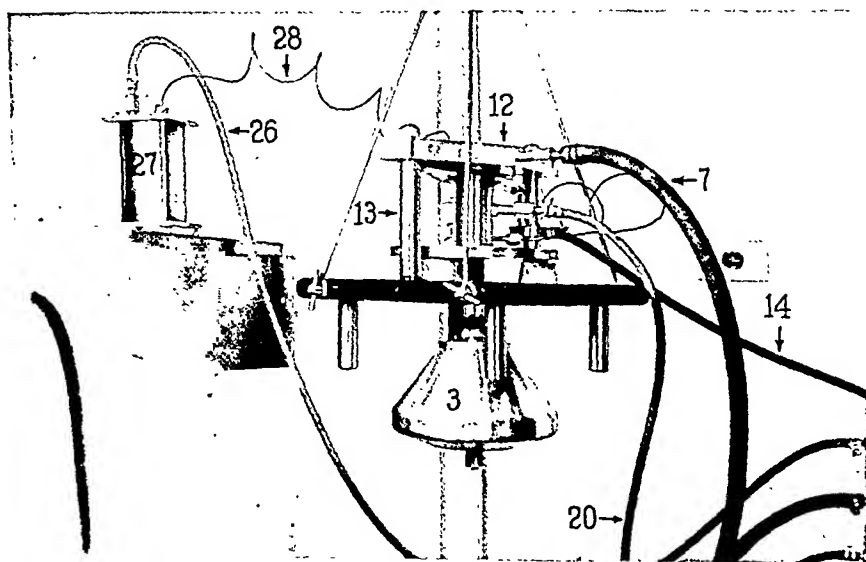


FIG. 5. Complete assembly of the driving mechanism and the rotor.

Fig. 1). After the bronze plugs are in place, a smooth bearing fit with the shaft is obtained by polishing the surfaces together with rouge. When finished, the fit should be such that the wire slips easily through the gland without excessive play.

Air pressure transmitted through the tubing (26, Fig. 5) forces lubricating oil from the container (27, Fig. 5) through the flexible copper tubing (28, Fig. 5) connecting to the coupling (29, Fig. 1) into the central cavity of the gland. The oil forms an air-tight seal about the shaft and also lubricates the two bearings. The lubricant escaping through the upper bearing (usually a fraction of a cubic centimeter per hour) is collected in a container (30, Fig. 1). The oil passing the lower bearing is allowed to fall into the vacuum chamber, although a collecting system such as described by Biscoe, Pickels, and Wyckoff (31) could be provided if necessary.

The vacuum seal is completed and the necessary flexibility provided by a section of soft, thick walled rubber tubing (31, Fig. 1) which joins the stem of the gland and a brass sleeve (32, Fig. 1) that is fastened and tightly sealed to the top plate (33, Fig. 1) of the chamber. Three screws clamp the air-bearing support and the oil gland to a circular brass plate (34, Fig. 1). This plate is supported by three soft rubber stoppers (35, Fig. 1) surmounted by small live rubber discs (36, Fig. 1).

Rotor.—The rotor which is shown in Figs. 6, 7, and 8 is so designed that while it rotates in a very high vacuum, the material which is being centrifuged is maintained under normal atmospheric pressure. It is machined from a solid block of duralumin alloy commercially known as ST 17. As shown in the illustrations (47, Figs. 6, 7, and 8), it is given a pear-shaped form having a maximum diameter of 8 inches and tapering off at the top to $3\frac{1}{2}$ inches. This form was chosen in order to reduce the weight of the rotor as much as possible without sacrificing its strength materially. At the top of the rotor there is a cavity (50, Fig. 6) cut to the depth of $1\frac{9}{16}$ inch. At the bottom edge of this cavity are drilled sixteen equally spaced holes, each $\frac{1}{2}$ inch in diameter and $2\frac{7}{8}$ inches deep, to accommodate the celluloid containers described below. The holes (48, Fig. 6) are inclined at an angle of 45° to the axis of rotation, and their bottoms are rounded to correspond to the shape of the containers. The distance from the inner edge of each hole to the axis of rotation is 3.8 cm. at the top, and 8.5 cm. at the bottom, where the outside supporting wall reaches a minimum thickness of $\frac{1}{8}$ inch. This wall is made considerably thicker at the top where the holes are close to each other and additional strength is needed. In Fig. 6, one container, 49, is indicated as being in position. The sixteen containers, each holding conveniently 7 cc. of fluid, give the centrifuge a total capacity of 112 cc. which can be centrifuged in one run. At the base of the rotor there is machined out a stem 1 inch in diameter and in length, which is used for fastening the rotor in the milling machine chuck while the holes are being drilled.

The rotor head (44, Figs. 6, 7, and 8) is machined from a separate piece of duralumin and is made to fit snugly into the top portion of the rotor. The stem (44a, Fig. 6) fits into a hole in the center of the rotor and aids in properly centering the rotor head when the latter is placed in position. A rubber washer (45, Figs. 6, 7) made of round rubber belting, $\frac{1}{8}$ inch in diameter and smeared with lubriscal,

is inserted in the step joint between the rotor head and the rotor proper to furnish an air-tight seal (Fig. 6). The rotor head is fastened to the rotor with six equally spaced screws (46, Figs. 6, 7). Fig. 8 shows the rotor complete. When assembled it weighs, without containers, 6,460 gm.

The rotor is fastened to the drive shaft with a special chuck arrangement attached to the rotor head as shown in detail in Fig. 9. The essential units of this arrangement are the steel chuck, 40, the duralumin screw cap, 41, three screws, 42, the steel ring, 43, and the steel bushing, 41a, which is forced into the screw cap. The chuck is closely fitted in the rotor head and is tapered and slotted (four cuts,

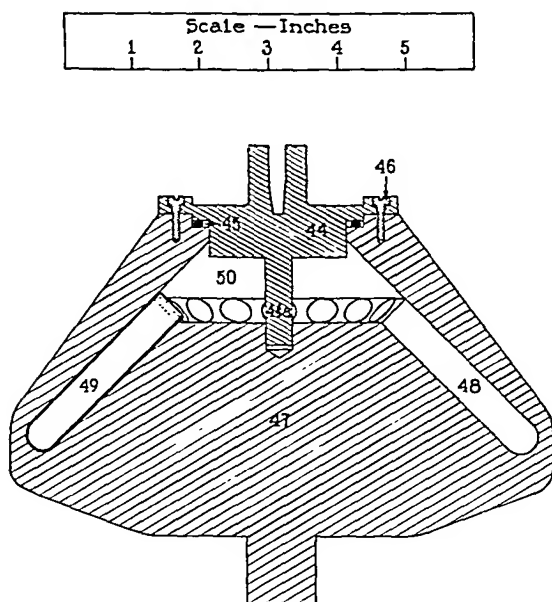


FIG. 6. Vertical cross-sectional view of the rotor.

40a, Fig. 9) at its lower end. The steel ring, 43, automatically raises the chuck and releases its hold on the shaft when the cap is unscrewed.

Containers.—Glass containers cannot be used in this centrifuge, as glass would be crushed under the centrifugal force necessary to separate viruses. The containers¹ are made of a celluloid composition, are transparent and flexible and, as shown in Fig. 10, have the shape of ordinary Wassermann tubes. They are manufactured in various sizes, but those used by us have a length of 75 mm., outside diameter of 13.2 mm., and a wall thickness of 0.4 mm. They weigh 1.35 gm. each, and have a total capacity of 8.5 cc. when filled to the rim. For centrifuging

¹ The most satisfactory containers for this purpose were found to be those manufactured by the Lusteroid Container Co., South Orange, New Jersey.

purposes 7 cc. of fluid can be safely placed in each without danger of spilling. As mentioned above, the bottoms of the holes in the rotor are reamed to correspond to the shape of the bottoms of the containers, which fit into the holes fairly snugly, leaving a minimum amount of empty space. When fitted in this manner and filled with fluid, these containers will withstand very high centrifugal forces—over 95,000 times gravity at the bottom portion—without breaking or leaking. These containers, holding 7 cc. of fluid and run for several hours at a speed of 25,000 R.P.M., remained unaltered; but when the speed exceeded 27,000 R.P.M., the empty portion at the top became bent and folded over the surface of the fluid, sealing it completely (see *A*, Fig. 10). When used only half filled, the empty portion of the tube usually collapses. The one serious disadvantage of these containers is that they cannot be sterilized in an autoclave or in a hot air sterilizer. They can be sterilized, however, either by boiling or by soaking in 75 per cent alcohol and subsequently by rinsing in sterile distilled water. They cost a little over one cent apiece and after being used once are usually discarded. It is not necessary to balance the containers by accurate weighing. In our experience it was sufficient to measure accurately with a pipette an equal amount of fluid into any two of the containers and to place them in the rotor in positions opposite to each other.

Vacuum Chamber.—The vacuum chamber in which the centrifuge rotates is made up of a cylinder and two flat plates as shown in Figs. 11 and 12. The cylinder (37, Fig. 11) is of chrome nickel steel alloy, S.A.E. 3140.² Its dimensions are: height, 11 inches; inside diameter, 9 inches; and wall thickness, 1 inch. After forging, it is machined to the desired shape and then heat treated. The heat treatment renders the steel so hard that it cannot be cut with ordinary lathe tools. This type of steel, having a very high tensile strength, was chosen to furnish safety provision in case of a rotor explosion at high speed. The top and bottom of the chamber (33, 33a, Fig. 11) are made of finished cold-rolled steel plate 12 inches square and 1 inch thick. At each of the four corners of these plates are inserted 1 inch stainless steel rods, each 3 inches long, to hold the cylinder in place. The sealing arrangement, which is the same at both the top and bottom of the cylinder, can be seen in Fig. 1. The two rubber washers (39, Figs. 1, 11) are made of round rubber belting $\frac{1}{2}$ inch in diameter, spliced and cemented together at the ends. When smeared with lubriseal, they furnish a satisfactory vacuum seal. A ring of sheet metal (38, Fig. 1) greatly facilitates the placing of the two washers in position and also removes the possibility of their slipping into the chamber. It is raised for placing the washers and is pushed into the indicated position (Fig. 1) by the top of the chamber. To the top plate is fastened the driving mechanism as described above. Through the bottom plate are bored two holes leading into the chamber. Into these are inserted brass sleeves to provide for rubber tubing connections, one of which leads to the vacuum pump, and the other to the vacuum gauge. The vacuum chamber is mounted on four rubber stoppers to absorb any vibration and rests on a concrete block 24 inches square and 6 inches thick, which

² Made for us by the Martin Forge Co. of Brooklyn.

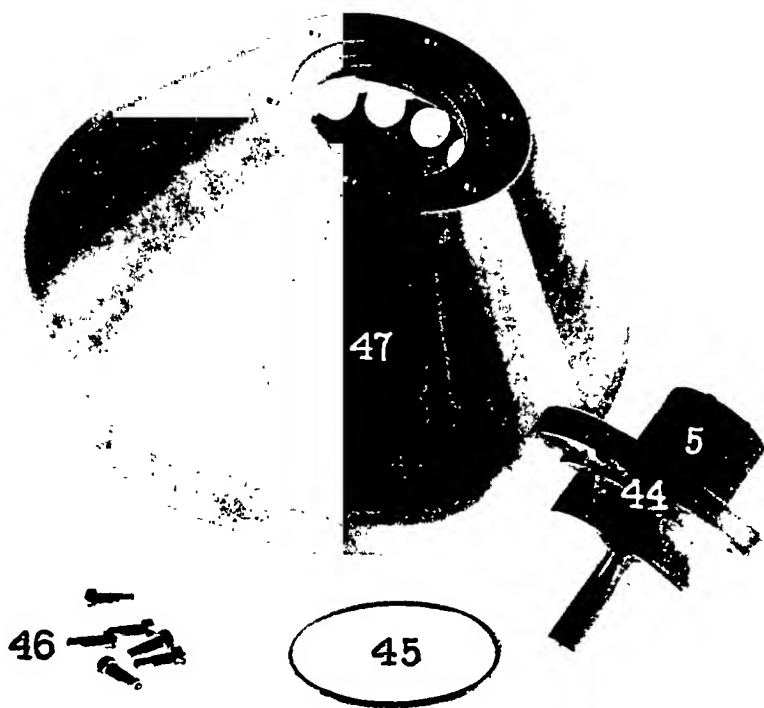


FIG. 7. Component parts of the rotor shown separately.

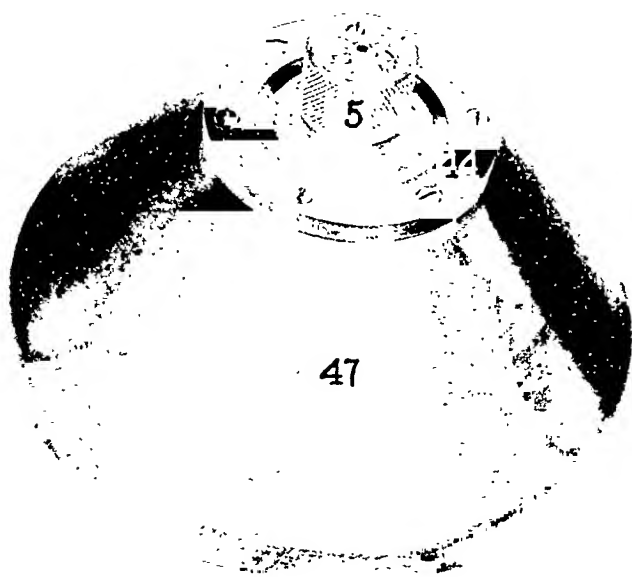


FIG. 8. Rotor shown assembled.

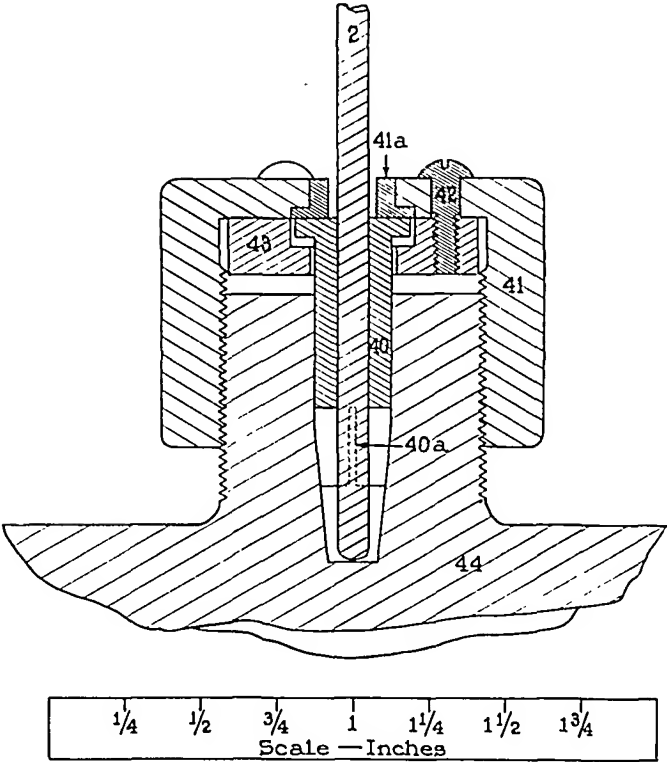


FIG. 9. Vertical cross-sectional view of the rotor chuck arrangement.

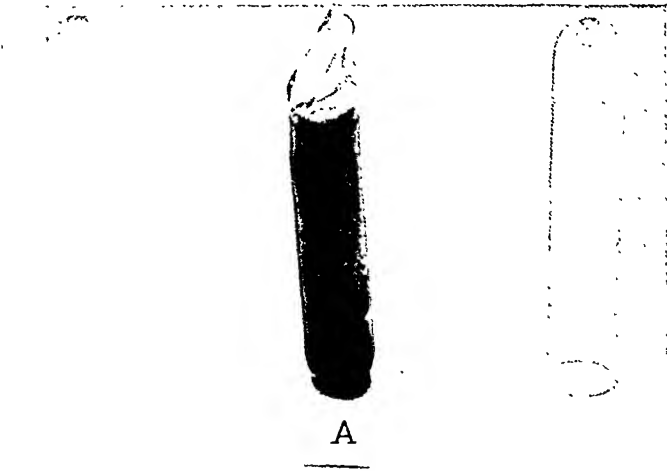


FIG. 10. Celluloid centrifuge tubes.

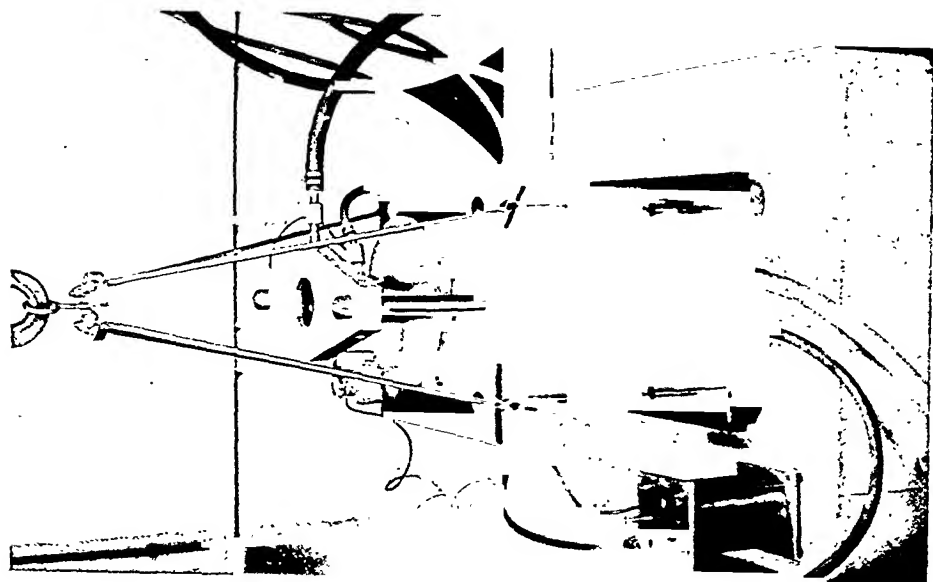


Fig. 12. Vacuum chamber closed.

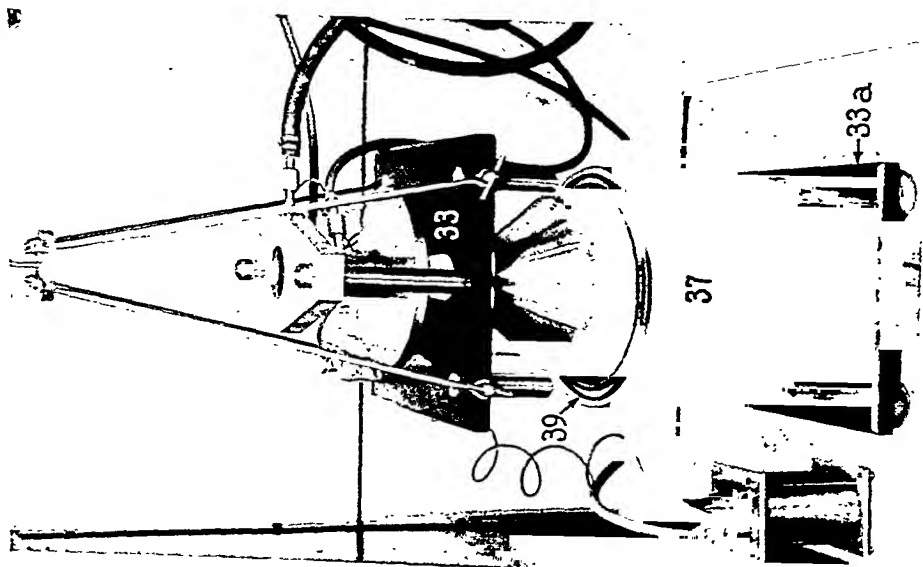


Fig. 11. Vacuum chamber open.

in turn rests on a layer of cork 3 inches thick. Fig. 12 shows the concrete block and the vacuum chamber closed.

Operation.—The accessories used in operating the centrifuge are pictured in Figs. 13 and 14. The air controls and the stroboscope for measuring speeds can be seen in Fig. 13. Fig. 14 shows, as they appear to the operator from the position of the controls and stroboscope: the hoist, 51, used to raise and lower the top of the vacuum chamber and the driving mechanism; the mirror, 52, used for viewing the turbine; the mercury manometer, 53, for measuring the pressure in the vacuum chamber; the vacuum pump, 54; and the barricade, 55, which is intended to serve as an additional protection to the operator in the event of a rotor explosion. This

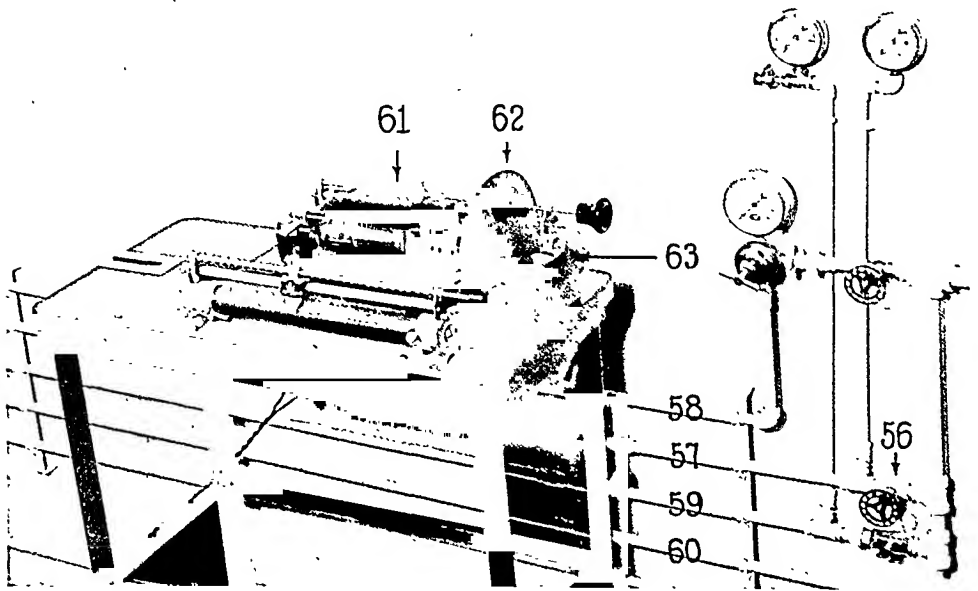


FIG. 13. Stroboscope and air controls.

barricade is a wooden box, 1 foot thick, which is filled with sand. Our vacuum pump is a high speed Cenco Megavac, but a smaller pump will serve the purpose, although the evacuation time preliminary to centrifuging is lengthened somewhat. A 60 watt lamp behind the barricade illuminates the turbine for stroboscopic.

Successful operation of the centrifuge depends upon having approximately constant pressures in all the air lines supplying the various parts of the driving mechanism. Since the head pressure in our laboratory is subject to considerable variation, the air is first passed through a two-stage reducing system of adjustable regulators. The first regulator drops the head pressure (150 to 225 pounds per square inch) to about 100 pounds per square inch. The second regulator reduces the pressure to 50 pounds per square inch and holds it constant at that value as long as the setting of the control valve (56, Fig. 13) for the driving line is not

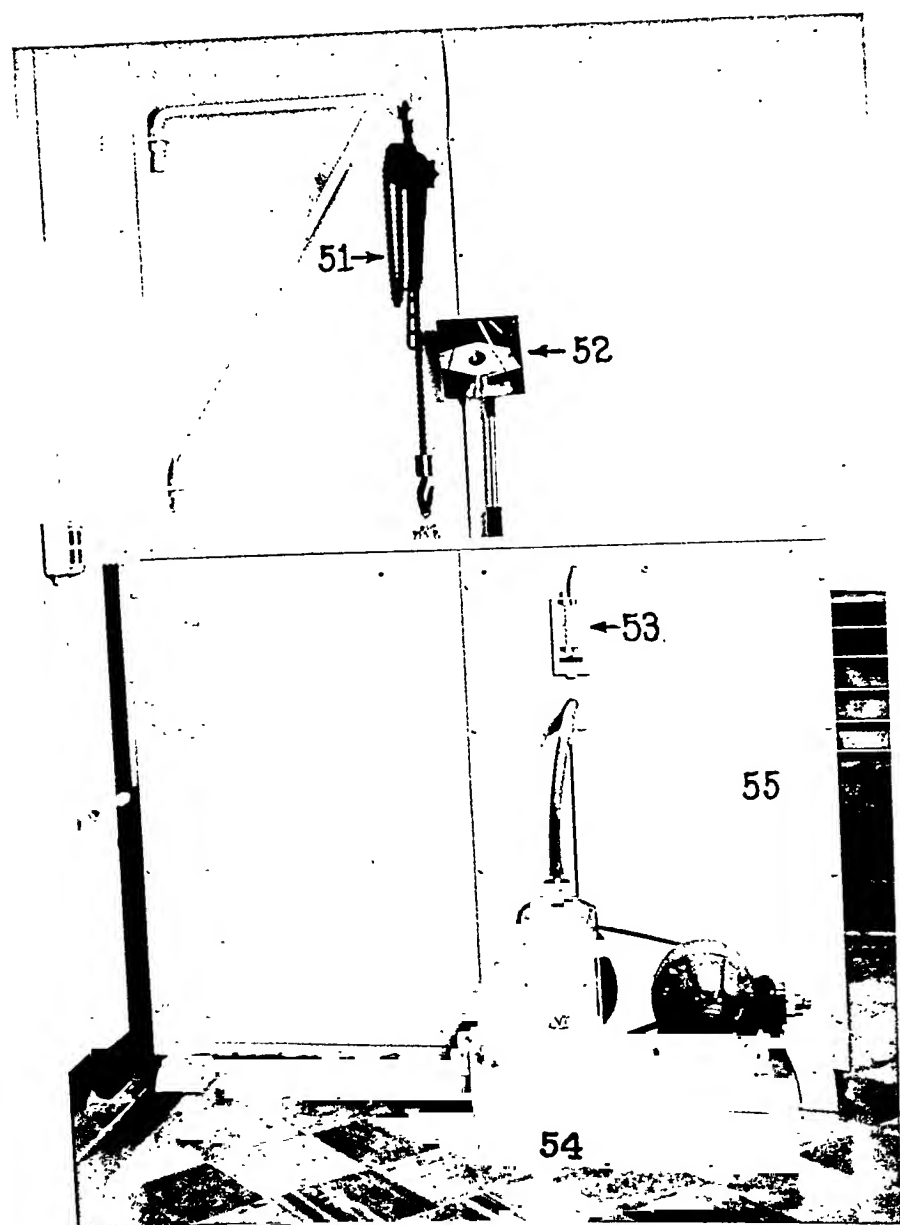


FIG. 14. Barricade, vacuum pump, gauge, hoist, and stroboscope mirror shown in position.

varied over too wide a range. Inexpensive regulators (Type 3Y)³ have proved satisfactory.

In Fig. 13 can be seen the air lines which lead to the centrifuge and connect with the flexible pressure tubings previously described. Line 57 supplies the driving jets. An ordinary needle valve, 56, gives sufficiently sensitive adjustment of the driving pressure. The gauge has a range of 0 to 60 pounds per square inch. Line 58 furnishes pressure for the oil container. This requires the least critical control and therefore either a small reducing valve or simply a needle valve and a small air leak serve very well. Any gauge which reads as high as 15 pounds per square inch is suitable. Air for the supporting bearing is supplied through line 59. It requires very critical adjustment and only a small flow of air. Consequently, a fine needle valve⁴ is used. The gauge range is 0 to 30 pounds per square inch. Air in line 60 operates the reversing mechanism and is controlled by a needle valve (not shown).

The speed attainable with the apparatus is limited only by the strength of the rotor, and since the explosion of a rotor run at excessive speed is both costly and dangerous, it is very important accurately to measure and control the speed. It is also important to know approximately what speeds can be regarded as safe for routine operations. The experiments of Biscoe, Pickels, and Wyckoff (32) on tensile strength and fatigue endurance of duralumin rotors furnish a practical basis for such approximation. It is estimated that the rotor described above would perhaps explode in the neighborhood of 40,000 R.P.M. Prolonged operation at a speed not exceeding 30,000 R.P.M., on the other hand, is considered comparatively safe if there are no serious flaws in the metal of which the rotor is made. In our experiments we have not exceeded this speed. The speed is regulated entirely by the adjustment of valve 56, in Fig. 13, and is measured frequently with the stroboscope, 61, which is of the simple motor-driven, slotted disc type. The operator views the spinning turbine, with the aid of the mirror mounted above the barricade, by sighting through the revolving slots, 62. A disc with 25 slots is appropriate for use with this particular centrifuge. Ours contains 50 slots because it is also used for higher speed ranges with the smaller rotors employed for molecular weight determinations. The motor is a $\frac{1}{4}$ horse power, 110 volt, direct current type. Its speed is controlled by two rheostats and is measured with a tachometer, 63, which is attached to the motor shaft with flexible rubber tubing. A stop watch is used for timing. It is highly desirable to have as steady a source of electric current as possible. To facilitate the synchronization of the centrifuge speed with the frequency of vision interruption, the turbine is first painted a dull black on its top surface and then has a white mark placed near the edge of this dark background. This mark appears to be stationary (although blurred) when synchronization is obtained. It should be remembered that such a single pattern can be obtained when the turbine speed is one, two, or three times the frequency of vision interrup-

³ Supplied by the Foster Engineering Co. of Newark.

⁴ Supplied by the Ashton Valve Co. of Boston.

tion. After practice, one can easily make distinctions by noting the amount of blurring. A variety of multiple patterns can also be obtained, and they serve as convenient checks on one another as well as on the single patterns. The double pattern, in which the turbine appears to have two opposite marks, is very convenient for measurement in the lower speed ranges because it is well defined and necessitates running the stroboscope motor at double speed and consequently more smoothly.

In the actual operation of the centrifuge the following procedure is generally used. After the containers with the material to be centrifuged are placed in the rotor, the rotor head is fastened in place and the assembled rotor fastened tightly to the drive shaft with a pin wrench. After the chamber top with the driving mechanism is lowered into place, the vacuum pump is started and is kept running throughout the experiment. The pressure in the oil container is set at approximately 10 pounds per square inch and the air-bearing pressure is raised until the turbine will coast freely when given a turn with the hand. The pressure required for this purpose with our present turbine and rotor is about $7\frac{1}{2}$ pounds per square inch. As soon as the vacuum gauge registers only a small fraction of 1 mm., the driving pressure is set at 35 pounds per square inch and maintained at this level until the desired speed is reached. About 35 minutes of acceleration are required to attain a speed of 30,000 R.P.M. Most of our centrifugation experiments with the yellow fever virus were carried out at a speed of 27,300 R.P.M. and after initial acceleration a driving pressure of 13 pounds per square inch was sufficient to maintain this speed fairly steadily. At times, however, a very slight acceleration was noted and further adjustments in the driving pressure were necessary in order to maintain the centrifuge at a constant speed. It should be noted that the rotor has a rather high inertia and therefore responds very slowly to small changes in the driving pressure.

In our early experiments the air filter (21, Fig. 1) was not used. Occasionally difficulty was encountered in getting the centrifuge started, and at times sudden drops in the speed occurred. The source of both troubles was traced to the braking action of grit or other small particles which had become lodged in the seat of the bakelite supporting ring. However, after the installation of the filter this source of trouble was eliminated. Difficulty may also be encountered if the bakelite ring is warped or improperly machined; in either case an examination will reveal that the ring is polished in two or more places instead of evenly all around.

At the start the rotor passes through a short period of rather pronounced precessional motions, but as it accelerates it soon settles into rotation about a fixed axis. Vibrations are perceptible in the oil gland and its supporting plate until the speed reaches about 10,000 R.P.M. With further increase in speed the motion gradually becomes extremely smooth with hardly a trace of vibration in any part. There are various critical speeds in the lower range at which vibration is quite pronounced, but the turbine and the rotor accelerate through these without ill effects. Undue vibration or shaking may be caused by: (a) a drive shaft of improper length; (b) a greatly unbalanced or misaligned rotor or turbine; (c) too

loose a fit in the bearings; (d) a bent drive shaft; (c) an improperly functioning air float; or (f) a lack of sufficient flexibility and damping in the mountings of the driving mechanism.

The air-bearing pressure sometimes has to be lowered very slightly as the rotor reaches higher speeds. A little experience with the peculiarities of this bearing makes the critical adjustment of its pressure comparatively easy. One soon becomes accustomed to the sounds emitted by the centrifuge and can readily tell by ear whether or not the valve can be better adjusted. While the speed is about 15,000 R.P.M., the operator can lightly touch the supporting plate (34, Fig. 1) with comparative safety and discover in that way the effects of slight readjustments in the needle valve controlling the support. Lowering the pressure too much causes the turbine to drag and decelerate. Generally the correct adjustment at any speed can be made by gradually increasing the pressure until the needle of the pressure gauge (in line 59, Fig. 13) begins to oscillate with a low period. At this stage the turbine may be seen to shift slightly from side to side in step with the needle oscillation. The pressure is then reduced just enough to eliminate completely these movements of the needle and the turbine. Runs of several hours' duration have been made without a single resetting of the air-bearing pressure.

A rapid stopping of the centrifuge is accomplished by opening the valve controlling the reversing mechanism and readjusting the driving pressure to the desired value; 35 pounds per square inch is sufficient to stop the rotor in about 20 minutes. The same vibrational periods occur during both deceleration and acceleration although the precessional motions are less pronounced in the slowing down.

If the reverse drive is not applied, and the rotor is allowed to coast until it comes to a standstill, it usually takes over 2 hours to decelerate from a speed of 30,000 R.P.M. to a full stop.

EXPERIMENTAL

A series of experiments was carried out with a view to studying the operating characteristics of the centrifuge as well as the behavior of yellow fever virus in an intense centrifugal field. In most of these a neurotropic strain of the virus derived from mouse brain was used. This was chosen because of its greater virulence for mice and its presence in the material under study is therefore more easily determined than that of the viscerotropic form derived from the blood of infected monkeys. As the virus becomes rapidly inactivated when suspended either in distilled water or in physiological saline, all the virus suspensions were prepared either in normal monkey serum or in ascitic fluid; also all dilutions in connection with the titrations of the virus were made in a diluent of similar nature. In each instance the

material was rendered clear, either by filtration or by preliminary centrifugation at lower speeds prior to its final centrifugation in the vacuum centrifuge. Various dilutions of the supernatant fluid and of the sediment after final centrifugation were tested in groups of six mice by intracerebral inoculations. The following experiments are described in detail as illustrative of the results obtained.

Experiment 1.—Five infected mouse brains were finely ground in a sterile mortar and were suspended in 20 cc. of a diluent consisting of 25 per cent normal

TABLE II

Experiment 1. Speed 25,000 Revolutions per Minute; Time 3 Hours

Material	Dilution	Mouse group	Results
Supernatant fluid	Undiluted	D 0429	All died
	10^{-1}	D 0430	3/5 "
	10^{-2}	D 0431	All lived
	10^{-3}	D 0432	" "
Sediment 1. Resuspended in 1 cc. of supernatant fluid	10^{-3}	D 0433	All died
	10^{-4}	D 0434	" "
	10^{-5}	D 0435	" "
	10^{-6}	D 0436	" "
	10^{-7}	D 0437	All lived
	10^{-8}	D 0438	" "
Sediment 2. Supernatant fluid poured off entirely and sediment suspended in 1.0 cc. of fresh diluents	10^{-3}	D 0439	All died
	10^{-4}	D 0440	" "
	10^{-5}	D 0441	" "
	10^{-6}	D 0442	4/5 "
	10^{-7}	D 0443	All lived
	10^{-8}	D 0444	" "

monkey serum in distilled water. This suspension contained approximately 7.5 per cent of the mouse-brain substance by weight. The mixture was first centrifuged for 30 minutes at a speed of about 3,000 R.P.M. and the supernatant fluid was then passed through a Seitz filter. The filtrate was placed in two sterile celluloid tubes, 7 cc. in each, and was centrifuged in the vacuum centrifuge for 3 hours at a speed of 25,000 R.P.M. (417 per second by stroboscopic measurement). From one of the two tubes 1.5 cc. of the topmost portion of the supernatant fluid was removed and was used for the titration of the virus content in mice; the middle portion was discarded and only 1 cc. was left at the bottom, in which the sediment was resuspended and designated as sediment 1. From the other tube the supernatant fluid was poured off entirely and the sediment which had been packed on

the bottom of the tube was resuspended in 1 cc. of a mixture consisting of 25 per cent ascitic fluid in distilled water. This was designated as sediment 2. Although the material before centrifugation was clear, both of the resuspended sediments were distinctly turbid. Serial tenfold dilutions were made from the portion of the supernatant fluid mentioned above as well as from both of the sediments, and groups of six mice were inoculated intracerebrally with each dilution. All dilutions were made in 25 per cent ascitic fluid and distilled water. The results which are shown in Table II indicate that effective separation of the virus had been obtained and that there was very little virus left in the supernatant fluid. The results further indicate that the virus apparently was packed on the bottom of the tubes to the extent that the supernatant fluid could be poured off and replaced with a new diluent without much loss of activity.

In order to determine the effect of the viscosity of the diluent on the rate of separation of the virus, the above experiment was repeated with a virus suspension which contained a much higher concentration of protein, as follows:

Experiment 2.—A 20 per cent mouse-brain suspension was prepared by grinding finely fifteen mouse brains in a sterile mortar and suspending them in 25 cc. of undiluted normal monkey serum. The suspension was centrifuged for 30 minutes at a speed of 3,000 R.P.M. The supernatant fluid was pipetted off and then was centrifuged for 1 hour at a speed of 13,000 R.P.M. After the second centrifugation the supernatant fluid was placed in two celluloid tubes, 7 cc. in each, and centrifuged for 3½ hours at a speed of 27,300 R.P.M. (455 per second by stroboscopic measurement) in the vacuum centrifuge. Along with the tubes containing the virus, two additional tubes containing water were placed in the centrifuge. At the end of the run the temperature of the water was measured with a thermometer and was found to be 1.2°C. higher than room temperature. After centrifugation the tubes containing the virus showed a definite whitish sediment at the bottom, and while most of the supernatant fluid was clear, the topmost layer of the column, about 5 mm. thick, showed a distinct grayish turbidity and apparently contained much finely dispersed fat. The supernatant fluid and the sediments were tested in mice in exactly the same manner as in the preceding experiment and the results are summarized in Table III. Although the material was centrifuged at a higher speed for a longer time than in the preceding experiment, the separation of the virus was less satisfactory. In fact, the supernatant fluid in this experiment proved still infective in a dilution of 1:10,000, while in the preceding test a 1:100 dilution gave entirely negative results.

Experiment 3.—It was difficult in Experiment 2 to understand the reasons for the relatively poor separation of the virus, which could not be explained by the higher viscosity of the diluent alone. Therefore it was decided to repeat the experiment in every detail with the exception that the material be centrifuged for a

longer period of time. Accordingly fifteen mouse brains were finely ground in a mortar and suspended in 25 cc. of normal monkey serum. After the suspension had been centrifuged for 30 minutes at 3,000 R.P.M. and for 1 hour at 13,000 R.P.M., the clear virus suspension was centrifuged for $5\frac{1}{2}$ hours at a speed of 27,300 R.P.M. After the completion of the run there was again present a layer of fat on the surface of the fluid similar to that observed in the preceding experiment. In addition, while most of the column of the fluid was reddish yellow in color due to the presence of hemoglobin, the topmost portion of the fluid had become perfectly

TABLE III

Experiment 2. Speed 27,300 Revolutions per Minute; Time $3\frac{1}{2}$ Hours

Material	Dilution	Mouse group	Results
Supernatant fluid	Undiluted	D 0703	All died
	10^{-1}	D 0704	" "
	10^{-2}	D 0705	" "
	10^{-3}	D 0706	2/6 "
	10^{-4}	D 0707	2/6 "
Sediment 1. Resuspended in 1.0 cc. of supernatant fluid	10^{-3}	D 0708	All died
	10^{-4}	D 0709	" "
	10^{-5}	D 0710	" "
	10^{-6}	D 0711	" "
	10^{-7}	D 0712	5/6 "
	10^{-8}	D 0713	3/6 "
Sediment 2. Supernatant fluid poured off entirely and sediment suspended in 1.0 cc. of fresh diluent	10^{-3}	D 0714	All died
	10^{-4}	D 0715	" "
	10^{-5}	D 0716	" "
	10^{-6}	D 0717	" "
	10^{-7}	D 0718	3/5 "
	10^{-8}	D 0719	All lived

colorless to a depth of about 1 cm., indicating a separation of hemoglobin from that portion. The supernatant fluid and the sediments were tested in animals precisely as in the preceding experiments. The sediment in both tubes was packed at the bottom so firmly that it was resuspended only with considerable difficulty; in fact, it was found impossible to break up some of the lumps. The results of the tests in mice are shown in Table IV. As seen from this table the effective separation of the virus was not much better than was observed in Experiment 2, although the centrifugation time had been prolonged 2 hours. The temperature of the rotor at the end of the run was not actually recorded, but it did not feel much warmer than room temperature. It was considered that the slight rise in the temperature that had taken place during centrifugation must

have been very gradual, and not sudden enough to set up temperature gradients and consequent convection currents which would have interfered with effective sedimentation of the virus. Moreover, there was evidence to indicate that separation of hemoglobin had taken place, which hardly could be expected had convection currents been present. It was felt, therefore, that if yellow fever virus particles are more or less of uniform size and have a density approximately that of protein, it was not the lack of sufficient centrifugal force which prevented complete separation of the virus, but the presence of other factors not clearly understood.

TABLE IV

Experiment 3. Speed 27,300 Revolutions per Minute; Time 5½ Hours

Material	Dilution	Mouse group	Results
Supernatant fluid	Undiluted	D 0895	All died
	10 ⁻¹	D 0896	" "
	10 ⁻²	D 0897	5/6 "
	10 ⁻³	D 0898	4/6 "
	10 ⁻⁴	D 0899	All lived
Sediment 1. Resuspended in 1.0 cc. of supernatant fluid	10 ⁻³	D 0900	All died
	10 ⁻⁴	D 0901	" "
	10 ⁻⁵	D 0902	" "
	10 ⁻⁶	D 0903	" "
	10 ⁻⁷	D 0904	2/6 "
	10 ⁻⁸	D 0905	3/5 "
Sediment 2. Supernatant fluid poured off entirely and sediment suspended in 1.0 cc. of fresh diluent	10 ⁻³	D 0906	All died
	10 ⁻⁴	D 0907	" "
	10 ⁻⁵	D 0908	" "
	10 ⁻⁶	D 0909	5/6 "
	10 ⁻⁷	D 0910	4/6 "
	10 ⁻⁸	D 0911	All lived

As mentioned above, in the last two experiments in which 20 per cent mouse-brain suspensions in undiluted monkey serum were used, there was in each instance a layer of finely dispersed fat present in the topmost portion of the fluid after centrifugation. This fat undoubtedly was derived from the brain tissue, although a small amount might have been present in the monkey serum. In all the experiments described above, the upper portion of the supernatant fluid, which was pipetted off and tested in mice for the presence of virus, contained most of the fat. It was considered a possibility that some of the virus particles might have been encapsulated in the fat globules and brought to the surface instead of being sedimented down under the centrifugal force. In order to determine this

possibility, the following experiment was carried out in which the fat from the mouse brains was extracted prior to use for centrifugation.

Experiment 4.—Dr. T. P. Hughes of this laboratory in his chemical studies on the virus found that the fat from infected mouse brains can be removed by extraction with ether without a material loss of the virus activity. He very kindly furnished us with 25 brains which had been dried *in vacuo* in the frozen state and from which the fat had been extracted in a Soxhlet apparatus with ether and petroleum ether. These brains were finely ground with quartz sand in a sterile mortar and suspended in 25 cc. of a diluent made up of equal parts of ascitic fluid and distilled water. As in the previous experiments, the material was rendered

TABLE V

Experiment 4. Speed 27,300 Revolutions per Minute; Time 3 Hours

Material	Dilution	Mouse group	Results
Supernatant fluid	Undiluted	D 1086	All died
	10 ⁻¹	D 1087	5/6 "
	10 ⁻²	D 1088	4/5 "
	10 ⁻³	D 1089	3/6 "
Sediment 1. Resuspended in 1.0 cc. of supernatant fluid	10 ⁻²	D 1090	All died
	10 ⁻³	D 1091	" "
	10 ⁻⁴	D 1092	" "
	10 ⁻⁵	D 1093	" "
	10 ⁻⁶	D 1094	" "
	10 ⁻⁷	D 1095	All lived
Sediment 2. Supernatant fluid poured off entirely and sediment suspended in 1.0 cc. of fresh diluent	10 ⁻²	D 1096	All died
	10 ⁻³	D 1097	" "
	10 ⁻⁴	D 1098	" "
	10 ⁻⁵	D 1099	" "
	10 ⁻⁶	D 1100	" "
	10 ⁻⁷	D 1101	1/6 "

clear by centrifuging first for 30 minutes at 3,000 R.P.M. and afterward for 1 hour at 13,000 R.P.M. The clear material was then centrifuged in the vacuum centrifuge for 3 hours at a speed of 27,300 R.P.M. The virus content in the supernatant fluid and sediments was tested in exactly the same manner as in the preceding experiments. The results are shown in Table V. As seen from this table there was still a considerable amount of virus present in the supernatant fluid, and in the titrations which were carried to a dilution of 1:1,000 the negative limit had not been reached. It is obvious from these results that factors other than the presence of fat are responsible for the failure to obtain a complete separation of the virus in the centrifuge.

In all these experiments the centrifuge was stopped by the application of the reverse air jets, and it usually required 20 minutes to decelerate from full speed to a full stop. It was considered another possibility that during the rapid deceleration a stirring and mixing may occur in the tubes, although the sharp boundary of the separated hemoglobin in Experiment 3 indicated against this. In order to obtain information on this point, another experiment was carried out in which fifteen mouse brains were finely ground with quartz sand and suspended in 25 cc. of a diluent containing 20 per cent normal monkey serum. After clarification by centrifuging at lower speeds, as in the preceding experiments, the virus mixture was again centrifuged for 3 hours in vacuum at a speed of 27,300 R.P.M. At the end of this period the driving pressure was cut off and the centrifuge was allowed to coast until it came to a standstill, which required slightly over 2 hours. As in Experiment 3, the top portion of the fluid showed a definite separation of the hemoglobin with a well defined boundary at about 1 cm. below the surface of the column of fluid. There was also a layer of fat accumulated on the surface, as observed in the preceding experiments. Titrations of the virus content of the supernatant fluid and of the sediment in mice gave similar results as observed in previous tests. Although the centrifuge was decelerated very slowly and there was evidence to indicate that a separation of hemoglobin in the top portion of the supernatant fluid had taken place, there was still a considerable amount of virus present in that portion of the fluid.

DISCUSSION

The centrifuge described above was successfully applied for the separation and concentration of yellow fever virus. The major portion of the virus was sedimented out of the suspension when centrifuged for 3 hours at a speed of 25,000 R.P.M.; in fact, it became so firmly packed to the bottom that the supernatant fluid could be poured off and the sediment resuspended in fresh diluent without appreciable loss of the virus activity. This would seem to indicate that the centrifuge can be applied for the washing and purification of the virus. At the speed of 25,000 R.P.M. the centrifugal force equals approximately 34,800 times gravity at the top of the fluid column and 66,000 times gravity at the bottom. However, a complete sedimentation of the virus was not obtained even when greater centrifugal forces were applied over a considerably longer period. A certain small proportion of the virus remained persistently in the supernatant fluid even when hemoglobin which was present in the virus suspension had been effectively separated. We are unable as yet to find a satisfactory explanation for the failure to secure a complete separation of

the virus under the conditions described, although we feel that this failure was not caused by convection currents or by stirring during deceleration. Investigations intended to determine the nature of this phenomenon are at present under way, but the results are not yet available.

Although we have not had an opportunity to study the behavior of other viruses in this centrifuge, we feel that it should prove a useful instrument in the study of filterable disease-producing agents, including the smallest known. It can be operated with comparative safety at speeds up to 30,000 R.P.M., giving a centrifugal force of approximately 50,000 times gravity at the top of the fluid columns and 95,000 times gravity at the bottom. Although the maximum hydrostatic pressure exerted by the fluid at this speed equals more than 600 atmospheres, the celluloid tubes have withstood this pressure well. The construction of the centrifuge is relatively simple and it could be built by any first class machinist. The cost of construction of the centrifuge itself, exclusive of the vacuum pump and other accessories, should not exceed the price of a standard laboratory centrifuge. In view of the fact that its air consumption is small and high pressures are not needed even for rapid initial acceleration, it could be operated in any laboratory where there is a supply of compressed air available.

SUMMARY

1. A high speed centrifuge is described in which the speed is limited only by the strength of the material of which the rotor is made. It carries sixteen tubes, each of which conveniently accommodates 7 cc. of fluid.
2. The centrifuge operates in a very high vacuum and therefore requires only a small amount of driving energy. The arrangement has been found to eliminate the possibility of producing injurious frictional heat.
3. The rotating parts are supported by an air-bearing and are driven by compressed air.
4. The centrifuge has been successfully operated at a speed of 30,000 revolutions per minute, representing a maximum centrifugal force in the fluid of 95,000 times gravity.
5. Celluloid tubes used for centrifugation of fluid at high speeds are described.

6. Experiments are described in which good sedimentation of the yellow fever virus was obtained.

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PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES: EFFECT OF BACTERIAL FILTRATES INJECTED INTRAVASCULARLY UPON REACTIONS TO ANTIGEN + ANTIBODY COMPLEXES

By GREGORY SHWARTZMAN,* M.D.

(From the Laboratories of The Mount Sinai Hospital, New York)

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It was previously reported that tissues prepared by bacteria or their soluble products undergo severe injury when acted upon by toxic principles resulting from intravascular interaction of animal protein antigens (*i.e.*, horse serum, egg albumin, etc.) with homologous antibodies. The interaction can be obtained in one of the following ways: By separate intravenous injections of the antigen and the antibody; by intravenous injection of the antigen into an animal possessing actively acquired homologous antibodies; by injection of the antigen into the prepared area with a simultaneous intravenous injection of the antibody; and by injection of the antigen into the prepared area in rabbits possessing actively acquired antibodies. In the latter case, there apparently occurs intravascular formation of the toxic principles at the site of the locally injected antigen with the circulating actively acquired antibodies. No similar reactions are obtained when normal rabbits (*i.e.*, those receiving no preparatory injection of bacterial filtrate) are injected intradermally either with the serum precipitinogen or the precipitating antiserum and after various intervals of time, reinjected intravenously with the precipitating antiserum in the case of the former, and with the serum precipitinogen in the case of the latter. Moreover, mixtures of serum precipitinogen with precipitating antiserum capable of eliciting severe hemorrhagic necrosis when injected intravenously, in skin sites prepared with a potent bacterial filtrate, consistently fail to prepare the skin for the provocative intravenous

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injection of bacterial filtrates. Thus, the antigen + antibody complexes, whilst endowed with the reacting potency described, are lacking in the skin-preparatory potency (Shwartzman (1)).

It was already shown that in the phenomenon of local skin reactivity to bacterial filtrates, the preparatory injection of the bacterial filtrate is made into the tissues (perivascular preparation), whilst the provocative injection is made intravascularly. In recent experiments it was found that the bacterial filtrates could also elicit the necessary preparatory effect by way of the vascular system provided the localization of the preparatory factors from the blood stream was allowed through the use of some accompanying agent (heat, Reynals' testicular extract) (Shwartzman (2)).

The purpose of the investigation embodied in this paper was two-fold: To determine whether an intravascular interaction of locally injected animal protein antigen with actively acquired antibodies is capable of eliciting the state of reactivity to bacterial filtrates; and also to determine whether bacterial filtrates injected intravascularly are capable of eliciting a local state of reactivity to antigen + antibody complexes interacting intravascularly.

EXPERIMENTAL

The Effect of Active Bacterial Filtrates upon Sites Injected with Horse Serum in Rabbits Sensitized to Horse Serum.—Before proceeding with the experiments summarized in Table I, it was important to ascertain again whether the normal horse serum employed possessed any skin-preparatory potency in normal rabbits (*i.e.*, those receiving no previous sensitizing injection of horse serum)

For this purpose, batches of rabbits were prepared by intradermal injection of 0.5 cc., 0.75 cc., and 1 cc. of normal horse serum, respectively. A group of ten rabbits was used for each of the above doses of horse serum. The rabbits of each group were further subdivided into subgroups A and B; subgroup A receiving 25 reacting units of meningococcus 44B. filtrate T.2041 intravenously, and subgroup B receiving 50 reacting units of the same filtrate. Most of the rabbits of subgroup A of each of the groups survived and showed no reactions. About 50 per cent of the rabbits of subgroup B died following the intravenous injection of the bacterial filtrate. One of the surviving rabbits showed a doubtful hemorrhagic reaction at the site prepared with 0.75 cc. of normal horse serum. The remaining rabbits were negative. It may be concluded that normal horse serum em-

ployed in these experiments, when injected intradermally even in amounts up to 1 cc., failed to elicit the state of reactivity to bacterial filtrates.

In the experiments described below rabbits received one sensitizing intravenous injection of normal horse serum in a dose of 1 cc., per kilo of body weight. The intradermal tests with horse serum were made 6 days after the intravenous sensitizing injection; rabbits thus sensitized and otherwise untreated gave no significant reactions outside of slight erythema and occasionally edema. As is well known, the Arthus phenomenon requires repeated sensitizations over a period of several weeks. 0.5 cc. was used for the test injections; preliminary experiments showed that doses smaller than 0.5 cc. may give inconsistent results. The above treatment was combined with injections of bacterial filtrates. The positive reactions described in this paper were hemorrhagic and necrotic lesions typical of the phenomenon of local skin reactivity to bacterial filtrates. They were considered prompt when they appeared 3 to 5 hours after the intradermal test injection with horse serum and delayed when they developed 24 hours later.

As is seen from Table I, sensitized rabbits receiving intravenous injections of bacterial filtrates simultaneously with the intradermal test with horse serum and 1 hour following it, showed no prompt local reactions (groups 1, 2, 3, 11, 12, and 16). In one rabbit, the intravenous injection of 75 units of meningococcus filtrate, given 1 hour after the intradermal test with horse serum, gave a delayed reaction (group 13). When the intravenous injection of bacterial filtrates was given 6, 18, and 24 hours following the intradermal injection of horse serum, hemorrhagic reactions appeared in the sites tested. The reactions appeared promptly in groups 4, 5, 6, 7, 8, 14, and 18. Exception was noted only in one rabbit (one delayed reaction, group 18). No reactions were obtained when the interval of time between the intravenous injection of bacterial filtrate and the intradermal test with horse serum exceeded 24 hours (groups 9 and 10).

As is seen, the intradermal tests with 0.25 cc. of horse serum (*i.e.*, group 19) in the sensitized rabbits gave no reactions, while 0.5 cc. of horse serum gave a reaction in 1 out of 21 rabbits tested (*i.e.*, group 20). Moreover, all the rabbits of groups 19 and 20 were also injected intravenously with 1 cc. of horse serum 24 hours after the intradermal tests (this part of the experiment not included in Table I). The intravenous injection produced no effect upon intradermal tests with 0.25 cc. In the group which received 0.5 cc. intradermally the subsequent intravenous injection enhanced the previously positive test in the rabbit described above and elicited a mildly hemorrhagic reaction in two rabbits at the site of previously negative intradermal tests. In one rabbit, the reaction appeared 4 hours, and in the second one 24 hours after the intravenous injection.

The following two explanations of the results of the experiments summarized in Table I suggest themselves. (a) Local state of reactivity may be elicited by the interaction of the locally injected horse serum with the circulating anti-horse antibodies of the sensitized

TABLE I

Reactions in Single Skin Sites Tested with Horse Serum in Rabbits Sensitized with Horse Serum and Injected Intravenously with Bacterial Filtrates Simultaneously and Following Skin Tests

Group No.	Sensitization with horse serum	Intra-dermal injection with horse serum	Intravenous injection		Interval of time between intradermal and intravenous injections hrs.	Readings of reactions 4 hrs. after intravenous injection	Readings of reactions 24 hrs. after intravenous injection	No. of reactions in each group intensified during 4 to 24 hrs. following intravenous injection
			Material	Dose				
1	cc.	cc.	T.2002 B.TyT _L *	25 reacting units	hrs.	0/5†	0/5	—
2	1	0.5	T.2007	25	—	0/4	0/4	—
3	1	0.5	T.2002	25	1	0/3	0/2(1 died)	—
4	1	0.5	T.2007	15	6	1/3	1/3	1
5	1	0.5	"	25	6	1/4	1/4	—
6	1	0.5	"	25	18	3/2	3/2	2
7	1	0.5	T.2002	25	24	5/10	3‡/5(7 died)	—
8	1	0.5	T.2007	25	24	4/13	3‡/7(7 died)	2
9	1	0.5	"	25	48	0/5	0/4(1 died)	—
10	1	0.5	"	25	96	0/5	0/3(2 died)	—
11	1	0.5	T.2017 Mg.44B§	25	1	0/3	0/3	—
12	1	0.5	T.2014	25	1	0/5	0/4(1 died)	—
13	1	0.5	"	75	1	0/3	1/2	1
14	1	0.5	"	25	24	3/1	1‡/1(2 died)	1
15	1	0.5	T.2041	25	24	2/1(1 died)	1/1(1 died)	—
16	1	0.5	"	50	1	0/3	0/3	—
17	1	0.5	"	25	20	3/2	3/2	—
18	1	0.5	"	10	20	1/4	2/3	1
19	1	0.25	—	—	—	0/9	0/9	—
20	1	0.5	—	—	—	1/20	1/20	—

* In this and the following tables, abbreviation B.TyT_L designates "agar washings" filtrates of *B. typhosus*, strain T^c cultures.

† In this and the following tables, the numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum of both indicates the total number of rabbits in each group.

‡ The reduction in number of rabbits is due to death 4 to 24 hours following the intravenous injection of bacterial filtrates.

§ In this and the following tables, abbreviation Mg.44B designates "agar washings" filtrates of meningococcus group III cultures.

rabbits. The reactive site thus obtained may then react with the bacterial filtrate injected intravenously after a suitable period of time. (b) It is also possible, however, that the preparatory factors of the bacterial filtrate injected intravenously localize at the site of the intradermal injection of horse serum in the sensitized rabbits, and then, in turn, induce at this site a state of reactivity to the interaction of locally injected horse serum with actively acquired anti-horse antibodies. As mentioned in the introduction, potent bacterial filtrates may produce a preparatory effect by way of the vascular system provided some local change raises the permeability of the blood vessels, thus allowing their passage into the perivascular tissue. It is significant in this connection that the incubation period necessary to induce the state of reactivity in this manner, may be less than 2 hours. It is possible, therefore, that the inflammatory reaction produced by the intradermal injection of the horse serum into sensitized rabbits may permit a localization of the sort with a subsequent elicitation of the state of reactivity to the reacting factors resulting from the antigen + antibody interaction.

In order to elucidate the mechanism of the reactions obtained in experiments of Table I, advantage was taken of the previous observations that the skin-preparatory potency of certain bacterial filtrates is not necessarily parallel to its reacting potency. Thus, quite frequently meningococcus culture filtrates may possess a high reacting potency with a low preparatory potency, whilst *B. typhosus* culture filtrates may have a skin-preparatory potency considerably higher than that of meningococcus with a reacting potency lower than that of meningococcus. This is illustrated in the titration of skin-preparatory factors against a constant reacting dose, as recorded in Table II.

In experiments recorded in Table III, rabbits were sensitized by an intravenous injection of 1 cc. of horse serum. With the exception of groups 9, 10, and 11 the period of sensitization was 6 days. Bacterial filtrates were injected intravenously preceding the skin tests at time intervals ranging from 1 to 96 hours (groups 1-7, 9-14, and 16-21). In groups 8 and 15, the bacterial filtrates were injected subcutaneously. Active and inactive bacterial filtrates were employed. Among the active ones there were those of both high skin-preparatory and provocative potencies (*B. typhosus* filtrates), those of high skin-preparatory potency and lesser provocative potency (meningococcus and *B. enteritidis* filtrates), and those with moderate provocative potency but inactive as skin-preparatory factors (*Streptococcus hemolyticus* and *B. tuberculosis* filtrates).

TABLE II
Titration of Phenomenon-Producing Polency of Bacterial Filtrates

Group No.	Titration of reacting factors		Provocative injection		Results	Mean concentration of reacting units per 1 cc.
	Skin-preparatory injection		Material	Dose		
	Material	Dose*				
1	T.2041 Mg.44B.	Undiluted	T.2041 Mg.44B.	1:3000	2/1	3500
2	"	"	"	1:4000	0/3	
3	T.2069 B.TyT _L	"	T.2069 B.TyT _L	1:900	2/1	
4	"	"	"	1:1100	0/3	1000
5	T.2006 B. enteritidis	"	T.2006 B. enteritidis	1:500	1/2	600
6	"	"	"	1:700	0/3	
Group No.	Titration of skin-preparatory factors		Provocative injection		Results	Mean concentration of preparatory units per 1 cc.
	Skin-preparatory injection		Material	Dose		
	Material	Dose				
7	T.2041 Mg.44B.	1:10	T.2041 Mg.44B.	25 reacting units	3/0	1000
8	"	1:50	"	25 "	2/1	
9	"	1:100	"	25 "	3/0	
10	"	1:200	"	25 "	2/1	
11	"	1:300	"	25 "	0/3	
12	"	1:800	"	25 "	0/3	
13	T.2069 B.TyT _L	1:50	"	25 "	2/1	
14	"	1:150	"	25 "	2/1	
15	"	1:600	"	25 "	3/1	

16	"	1:1000	"	25	1/1	5000
17	"	1:1500	"	25	0/3	
18	"	1:50	"	25	1/2	300
19	T.2005 <i>B. enteritidis</i>	1:100	"	25	0/3	
20	T.2010 human Tb. filtrate	Undiluted	T.2041 Mg.44B.	25	0/3	
21	T.2041 Mg.44B.	1:10	T.2010 human Tb. filtrate	25	0/3	
22	T.2025 <i>Streptococcus hemolyticus</i>	Undiluted	T.2041 Mg.44B.	2 cc. 25 reacting units	0/3	
23	21886 T.2041 Mg.44B.	1:3	T.2025 <i>Streptococcus hemolyticus</i> 21886	2 cc.	2/1	

* 0.25 cc. was injected intradermally.

TABLE III

Reactions in Single Skin Sites Tested with Horse Serum in Rabbits Sensitized with Horse Serum and Treated with Bacterial Filtrates
Prior to the Skin Tests

Group No.	Sensitization with horse serum	Period of sensitization days	Injection of bacterial filtrates		Route of injection	Dose of undiluted normal horse serum cc.	Interval of time between injection of bacterial filtrates and intradermal test hrs.	Readings of reactions 4 hrs. after intradermal injection	Readings of reactions 24 hrs. after intradermal injection	No. of reactions in each group intradermal injected during 24 hrs. following
			Material	Dose						
1	1	6	T.2007 B.TyT _L	5 reacting units	IV	0.5	1	0/5	2/3	2
2	1	6	T.1986 "	25 "	"	0.5	1	0/3	3/0	3
3	1	6	T.2041 Mg.44B.	5 "	"	0.5	1	0/4	0/3(1 died)	—
4	1	6	T.1968 "	25 "	"	0.5	1	0/5	0/4(1 died)	—
5	1	6	T.2041 "	25 "	"	0.5	1	0/3	1±/3	1?
6	1	6	" "	50 "	"	0.5	1	0/12	1±/11(1 died)	—
7	1	6	" "	75 "	"	0.5	1	2/0	2/0	2
8	1	6	T.2002 B.TyT _L	25 "	SC	0.5	1	0/3	0/3	—
9	1	2	" "	25 "	IV	0.5	1	0/6	0/6	—
10	1	3	" "	25 "	"	0.5	1	0/3	0/3	—
11	1	4	" "	25 "	"	0.5	1	0/3	0/3	—
12	1	6	" "	25 "	"	0.5	6	0/4	0/3	—
13	1	6	" "	15 "	"	0.5	18	0/4	0/3(1 died)	1
14	1	6	" "	25 "	"	0.5	24	3/1	1/1	1
15	1	6	" "	25 "	"	0.5	24	0/4	2/1(1 died)	—
16	1	6	T.2007 "	25 "	SC	0.5	48	1±/3	0/4	—
17	1	6	" "	20 "	IV	0.5	96	0/3	1±/3	—
18	1	6	T.2005 <i>B. enteritidis</i>	25 "	"	0.5	1	0/4	0/3	—
19	1	6	T.2010 human Tb. filtrate	2 cc.	"	0.5	1	0/6	0/4	—
20	1	6	T.2025 <i>Streptococcus hemolyticus</i> 21886	2 "	"	0.5	1	0/4	0/6	—
21	1	6	T.2009 <i>Streptococcus hemolyticus</i> 74246	1 "	"	0.5	1	0/3	0/4	—

IV = intravenous; SC = subcutaneous.

When the intravenous injection of *B. typhosus* "agar washings" filtrates (5 to 25 units) preceded by 1 hour the intradermal test with horse serum in rabbits sensitized to horse serum, there developed severe but delayed local reactions (groups 1 and 2). No reactions were obtained in sites of horse serum tests if preceded 6 hours before by the intravenous injection of *B. typhosus* "agar washings" filtrates in a dose of 25 units (group 12). When the *B. typhosus* filtrate was injected 18 and 24 hours prior to the test, there appeared delayed and also some prompt reactions (groups 13 and 14, respectively).

With longer intervals of time between the injections of the bacterial filtrate and the intradermal tests with horse serum, no reactions appeared (48 hours—group 16, and 96 hours—group 17).

As may be seen from the data thus far presented, filtrates endowed with reacting potency but devoid of skin-preparatory factors fail to elicit the reactions. When *B. enteritidis* filtrates possessing both skin-preparatory and reacting potencies are employed, the dilutions representing 15 skin-preparatory doses also fail to elicit the reactions in spite of the fact that they may contain as many as 25 reacting units. Convincing results, however, are afforded by comparison of the effect of *B. typhosus* and meningococcus filtrates. As is seen from Table II, the skin-preparatory potency of *B. typhosus* is approximately five times higher than that of meningococcus filtrate. In contrast, the reacting potency of *B. typhosus* filtrate is three and one-half times lower than that of meningococcus. Nevertheless, irrespective of the concentration of reacting units, the dilutions employed are effective only provided they contain a certain minimal amount of skin-preparatory factors. Thus, for example, the dilution 1:70 of meningococcus filtrate containing approximately 15 skin-preparatory units and 50 reacting units produces no effect. In order to obtain the reactions it is necessary to employ a dilution of 1:45 which has about 23 skin-preparatory units and 75 reacting units. On the contrary, the dilution 1:200 of *B. typhosus* filtrate containing 25 skin-preparatory units and only 5 reacting units is already sufficient to elicit strong reactions. It is obvious, therefore, that the ability of the bacterial filtrates to elicit reactions in antigen-injected sites is strictly conditioned by their skin-preparatory potency and seems to be independent of the concentration of reacting units.

It may be also noted from Table III that subcutaneous treatment of rabbits with bacterial filtrates 1 hour prior to the test remains

TABLE IV

Reactions in Two Skin Sites Tested with Horse Serum in Rabbits Sensitized with Horse Serum and Treated with Bacterial Filtrates

Group No.	Sensitization with horse serum		Period of sensitization		Intradermal injections with horse serum		Interval of time between intradermal injections	Intravenous injection		Order of injections	Interval of time between 1st intradermal and intravenous injection	Readings of reactions 4 hrs. after intravenous injection		Readings of reactions 24 hrs. after intradermal injection		Death
	cc.	days	1	2	cc.	cc.		Material	Dose			Site 1	Site 2	Site 1	Site 2	
1	1	6	0.5	0.5	cc.	0.5	Simultaneous	T.2002 B.TyT _L	25 reacting units	IV, ID	hrs. 1	0/6	0/6	0/5	0/5	1
2	1	6	0.25	0.25		0.25	"	T.2007	25 "	"	1	0/6	0/6	0/4	0/4	2
3	1	6	0.25 dil. 1:5	0.25 dil. 1:5		0.25 dil. 1:5	"	"	25 "	"	1	0/5	0/5	0/3	0/3	2
4	1	6	0.1	0.1		0.1	"	"	25 "	"	1	0/5	0/5	0/4	0/4	1
5	1	6	0.25	0.25		0.25	"	T.2081	25 "	ID, IV	24	1/2	1/2	1/2	1/2	—

ID = intradermal; IV = intravenous.

without effect; and also that period of sensitization of horse serum shorter than 6 days is insufficient.

In the work described thus far, single sites were tested intradermally with horse serum. In the following experiments, rabbits sensitized to horse serum received simultaneously two intradermal injections of horse serum. Bacterial filtrates were injected prior to and following the test injections with horse serum.

In groups 1-4 of Table IV, the intravenous injection of 25 reacting units, per kilo of body weight, preceded these tests by 1 hour. No reactions were obtained, whilst positive reactions were produced in sites of single tests of otherwise similar experiments (groups 1 and 2 of Table III).

Two simultaneous injections of horse serum preceding the injection of bacterial filtrate by 24 hours, yielded reactions similar to the single site tests (group 5 of Table IV compared with groups 7, 8, 14, and 15 of Table I). This incidental observation is possibly related to some process of local desensitization to the anaphylactic reaction with horse serum.

Effect of Intravenous Injection of Antigen + Antibody Complexes upon Skin Tests with Horse Serum in Rabbits Sensitized to Horse Serum.—As previously reported, whilst certain bacterial filtrates alone are capable of eliciting a state of reactivity, potent provocative factors may be obtained also from combinations of animal protein antigens with homologous antibodies. Advantage was taken of this observation in order to substantiate further the above conclusion that the ability of a bacterial filtrate injected intravenously to produce reactions at sites with normal horse serum in sensitized rabbits is strictly conditioned by its skin-preparatory potency.

Antigen + antibody complexes employed were mixtures of anti-human horse serum with human serum made in proportions yielding potent reacting factors. In groups 6, 7, and 8, normal rabbits were prepared by intradermal injections of 0.25 cc. of meningococcus group I (strain 44D.) "agar washings" filtrates diluted 1:2. Rabbits of group 9 were sensitized 6 days previously by an intravenous injection of 1 cc. horse serum and prepared by intradermal injection of 0.25 cc. of meningococcus group III (strain 44B.) "agar washings" filtrates diluted 1:2. 24 hours after the preparatory intradermal injections rabbits of groups 7-9 received the above mixture of human serum with anti-human horse serum.

As is seen from Table V, the antigen + antibody mixtures were endowed with strong reacting potency demonstrable both in normal and in horse serum-sensitized rabbits. As may be also seen from the experiment of group 10, the same mixtures were totally devoid of skin-preparatory potency.

TABLE V
Effect of Reactions in Single Skin Sites Tested with Horse Serum in Rabbits Sensitized with Horse Serum and Treated with Combinations of Protein Antigens and Homologous Antibodies

Group No.	Sensitization with horse serum	Period of sensitization	Intradermal injection	Intravenous injection		Order of injections	Interval of time between intradermal and intravenous injections	Readings of reactions 4 hrs. after intradermal injection	Readings of reactions 4 to 24 hrs. after intradermal injection
				Material	Dose*				
1	1	6	0.5 cc. horse serum	Equal parts of anti-human horse serum H52 + horse serum dil. 1:20	2	IV, ID	1	0/3	0/3
2	1	6	0.5 " "	Equal parts of anti-human horse serum H52 + horse serum dil. 1:16	2	" "	1	0/4	0/4
3	1	6	0.5 " "	Equal parts of anti-human horse serum H606 + human serum dil. 1:8	2	ID, IV	24	0/5	0/5
4	—	—	T.2053 Mg.44D ₁ dil. 1:2	Equal parts of anti-human horse serum H592 + human serum dil. 1:16	2	" "	24	2/0	2/0
5	—	—	" "	Equal parts of anti-human horse serum H592 + human serum dil. 1:20	2	" "	24	2/0	2/0
6	—	—	" "	Equal parts of anti-human horse serum H606 + human serum dil. 1:8	2	" "	24	2/1	2/1
7	1	6	T. 2041 Mg.44B. dil. 1:2	" "	2	" "	24	3/0	3/0

ID = intradermal; IV = intravenous.

* All doses calculated per kilo of body weight.

After ascertaining the reacting potency of the antigen + antibody complexes, it was attempted to determine the effect of these complexes upon skin sites injected with horse serum in rabbits sensitized to horse serum 6 days previously. As was noted (Tables I and III), the intravenous injection of potent bacterial filtrates preceded by 1 hour or following after 24 hours, the intradermal tests with horse serum, invariably resulted in prompt reactions at sites of the tests with horse serum in rabbits sensitized to horse serum. In the experiments of groups 3, 4, and 5 of Table V, the injection of bacterial filtrates prior to the test with horse serum, or following it, was substituted by the above described antigen + antibody mixtures of ascertained reacting potency. No reactions resulted in these experiments.

Effect of Bacterial Filtrates Injected Intravascularly upon Reactions to Passively Acquired Antigen + Antibody Complexes.—Inasmuch as all the experiments about to be described remained consistently negative, only a brief summary is given below.

Series 1.—Rabbits received the antigen (human serum) intravenously in a dose of 2 cc., per kilo of body weight, and 1, 6, and 23 hours later were injected intravenously with 25 reacting units of *B. typhosus* "agar washings" filtrates. 24 hours after the injection of the human serum (*i.e.*, 1, 18, and 23 hours after injection of the bacterial filtrate, respectively), the animals received a single intradermal injection of 0.5 cc. of anti-human-horse serum.

Series 2.—Rabbits received the antibody (anti-human horse serum) intravenously in a dose of 5 cc., per kilo of body weight, and 1, 6, and 23 hours later, were injected intravenously with 25 reacting units of *B. typhosus* "agar washings" filtrates. 24 hours after the injection of the antibody, the animals received a single intradermal injection of 0.5 cc. of human serum.

Series 3.—In group A of this series, rabbits received human serum intravenously in a dose of 2 cc., per kilo of body weight; in group B, the animals received anti-human horse serum in a dose of 5 cc., per kilo of body weight. After intervals of 1, 6, and 23 hours rabbits of both groups were injected with mixtures of human serum and horse serum in a proportion giving rise to a heavy precipitate. Group A received intradermally anti-human horse serum 24 hours after the intravenous injection of the human serum. Group B received intradermally 0.5 cc. of anti-human horse serum 24 hours after the intravenous injection of human serum.

Series 4.—In this series an intravenous injection of *B. typhosus* "agar washings" filtrates in a dose of 25 reacting units, per kilo of body weight, preceded the injections of anti-human, horse, and human sera. The order of injections of the sera varied in the same manner as in experiments of series 1, 2, and 3.

TABLE VI

Methods	Elicitation of state of reactivity		Production of reaction in reactive sites		Results
	Preparatory factors	Mode of introduction	Provocative factors	Mode of introduction	
1	Bacterial filtrates; bacterial infections; vaccinia virus	Local perivascular preparation (dermis, parenchyma of internal organs, serous and mucous membranes)	Bacterial filtrates	General vascular route	Positive
2	Bacterial filtrates	Local perivascular preparation (dermis, parenchyma of internal organs)	Bacterial filtrates	Local perivascular route	Negative
3	Bacterial filtrates	Local perivascular preparation (dermis)	Inert colloids (silica, gelatine, etc.)	General vascular route	Negative
4	Bacterial filtrates	Local perivascular preparation (dermis)	Agar and starch	General vascular route	Irregular*
5	Bacterial filtrates	Local perivascular preparation (dermis)	Antigen + antibody complexes (animal protein antigens + antisera; inactive bacterial antigens + antisera); mixtures <i>in vitro</i>	General vascular route	Positive
6	Bacterial filtrates	Local perivascular preparation (dermis)	Antigen + antibody complexes (animal protein antigens + antisera; inactive bacterial antigens + antisera); <i>in vivo</i> interaction	Antigen introduced into general vascular system of actively sensitized rabbit	Positive

7	Bacterial filtrates	Local perivascular preparation (dermis)	Antigen + antibody complexes (animal protein antigens + antisera; inactive bacterial antigens + antisera); <i>in vivo</i> interaction	Antigen introduced into skin site prepared with bacterial filtrates in actively sensitized rabbit	Positive
8	Bacterial filtrates	Local perivascular preparation (dermis)	Antigen + antibody complexes (animal protein antigens + antisera; inactive bacterial antigens + antisera); <i>in vivo</i> interaction	Antigen introduced into general vascular system of passively sensitized rabbit	Positive
9	Bacterial filtrates	Local perivascular preparation (dermis)	Antigen + antibody complexes (animal protein antigens + antisera; inactive bacterial antigens + antisera); <i>in vivo</i> interaction	Antigen introduced into skin site prepared with bacterial filtrates in passively sensitized rabbit	Positive
10-18	Non-bacterial inflammatory and irritating substances; mixtures of animal protein with antisera; mixtures of inactive bacterial products with antisera; pneumococcus carbohydrates with precipitating antisera	Local perivascular preparation (dermis)	The same as 1-9	The same as 1-9	Negative

* In recent extensive experiments reactions were not obtained with the same regularity as with potent bacterial filtrates and antigen + antibody complexes.

TABLE VI—Concluded

Methods	Elicitation of state of reactivity		Production of reaction in reactive sites		Results
	Preparatory factors	Mode of introduction	Provocative factors	Mode of introduction	
19	Bacterial filtrates	Local vascular preparation (renal artery)	Bacterial filtrates	General vascular route	Positive
20	Bacterial filtrates	Local vascular preparation (marginal vein of clamped off ear)	Bacterial filtrates	General vascular route	Negative
21	Bacterial filtrates, in combination with: cold, xylol, ethyl urethane, pilocarpine, atropine, calcium gluconate, liver extract, histamine, adrenaline, and pituitrin	Local vascular preparation (marginal vein of clamped off ear)	Bacterial filtrates	General vascular route	Negative
22	Bacterial filtrates, in combination with: heat; testicular extract	Local vascular preparation (marginal vein of clamped off ear)	Bacterial filtrates	General vascular route	Negative
23	Bacterial filtrates	General vascular preparation	Bacterial filtrates	General vascular route	Clearly positive in kidneys only
24	Bacterial filtrates	General vascular preparation	Antigen + antibody complexes (animal protein antigens + antisera); <i>in vivo</i> interaction	Antigen intradermally in actively sensitized rabbit	Positive
25	Bacterial filtrates	General vascular preparation	Antigen + antibody complexes (animal protein antigens + antisera); <i>in vivo</i> interaction	Antigen intradermally in passively sensitized rabbit	Negative

DISCUSSION

The investigations reported in this and previous papers demonstrate the possibility of reproducing the phenomenon of local tissue reactivity to bacterial filtrates in a number of different ways (Table VI).

It should be strongly emphasized, however, that the following essential characteristics are used as criteria for recognition of the phenomenon under discussion.

1. The direct effect of the material used for the preparatory injection is insignificant in the gross and completely out of proportion to the type and severity of reaction obtained following the intravenous injection of potent provocative factors.

2. There exists no correlation between the intensity of direct reaction to the preparatory injection and the severity of the reaction following the injection of the provocative factors.

3. The typical reaction of the phenomenon in the gross observed after the provocative injection is essentially of a hemorrhagic type with incidental extensive necrobiosis and subsequent inflammatory reaction of tissues adjacent to necrotic sites. The reaction is first recognized by the appearance of scattered petechiae which promptly become confluent and within a few hours involve the prepared site with hemorrhage and necrosis of uniform severity. The lesion is flat or only slightly raised provided the preliminary treatment does not elicit any edema. In the gross, this picture is clearly different from the Arthus phenomenon. The reaction of the latter is essentially inflammatory in nature. During the first weeks of sensitization the inflammation is mild, expressing itself in swelling and redness. The sixth or seventh and subsequent weekly test injections result in intense reactions consisting of sizable red swellings with central zones of hemorrhage and necrosis. The reactions develop fully, usually 24 hours after the test injection.

As may be seen from Table VI, whilst it is essential that the reacting factors be present in the general circulation, the preparatory effect may be elicited in one of the three following ways.

1. Preparatory injection of active bacterial filtrate into the skin or parenchyma of internal organs (local perivascular preparation); dermis, lung (Shwartzman (3)); liposarcoma of guinea pig (Gratia

and Linz (4)); stomach (Karsner, Ecker, and Jackson (5)); knee joint (Moritz and Morley (6)); appendix (Latteri (7)); kidney (Loi and Cardia (8)); adrenal (Gronchi (9)); conjunctiva (Cassuto (10)); pancreas (Reitano and Lui (11)); etc.

The optimum interval of time between the preparatory and provocative injections is 24 hours, although in some rabbits an interval as short as 8 hours may be sufficient. The duration of reactivity induced depends on the potency and dose of the bacterial preparation and ordinarily disappears within 48 to 96 hours.

2. Injection into the vascular system of an organ (local vascular preparation). In 1929 the following experiment was performed in collaboration with Baehr (Shwartzman (3)).

Kidneys of rabbits were exposed by median laparotomy. The left renal vein was clamped off and 0.5 cc. of *B. typhosus* "agar washings" filtrate was injected into the left renal artery. The clamp was released 5 minutes following the injection. The right kidney received an injection of phenolized saline solution under the same conditions. 24 hours later *B. typhosus* "agar washings" filtrate, in a dose of 100 reacting units, per kilo of body weight, was injected into the ear vein. 24 hours after the provocative injection the left kidneys showed severe hemorrhagic and necrotic lesions of the cortex and medulla. No gross lesions were observed in the right kidney. Systematic histological studies were not made at the time. It was concluded from these experiments that the preparatory factors are capable of eliciting a state of reactivity by way of the vascular system of the kidney.

Inasmuch as the vascular system of the kidney possesses an unusually high degree of permeability which may be altogether different from other organs, it was of interest to determine whether a similar mode of preparation could be successfully obtained in the rabbit's ear. It was found that the state of reactivity could not be elicited by a preparatory intravenous injection of bacterial filtrates alone into clamped off and nonclamped ears. The state also failed to appear in combination with cold, xylol, ethyl urethane, pilocarpine hydrochloride, atropine, calcium gluconate, guinea pig liver extract, histamine dihydrochloride, adrenalin chloride, and pituitrin.

Preparatory intravenous injections of active principles, however, were capable of eliciting the state of reactivity in the rabbit's ear when they were accompanied by thermal hyperemia (*i.e.*, exposure

to 45°, 50°, and 55°C.). It was also possible to induce the state of reactivity when a mixture of the preparatory factors with testicular extract was given into the veins of clamped off ears. The incubation period required may be less than 2 hours (Shwartzman (2)).

As is seen from the macroscopic and microscopic studies on the phenomenon, in all probability the state of reactivity takes place in the elements of the terminal vascular network of the tissue prepared, after a suitable incubation period. Under ordinary conditions of intravascular preparation the contact of the preparatory factors with the vascular wall lasts only as long as the circulation is stopped and probably ceases when it is re-established. If, however, a state of enhanced permeability is induced by means of testicular extract (Reynals factors), a diffusion of the bacterial factors is allowed from the vascular channels into the perivascular tissue. A perivascular depot of active principles thus created may, then, induce the state of reactivity through a contact with the local vascular network for the necessary length of time.

The natural high permeability of the kidney vascular supply may allow the perivascular deposition of preparatory factors from the circulating blood, making it possible to elicit the state of reactivity by way of the local vascular system without the use of additional agents.

3. Preparation of organs by a preparatory injection of the bacterial filtrate into the general circulation.

Gratia and Linz (12) injected two doses of bacterial filtrate intravenously 24 hours apart and observed hemorrhages in the internal organs in guinea pigs and rabbits. Subsequently, Apitz (13) found that a single dose of filtrate, when given intravenously, produced similar changes in the lungs, liver, and spleen, but that lesions characteristic of the phenomenon were obtained in the kidneys only when the animals received two doses, 24 hours apart.

Histologically, the renal alterations consisted of localized or diffuse symmetrical cortical necrosis with arterial necrosis and thrombosis, focal glomerular capillary thrombosis and necrosis of adjacent tubules, interstitial hemorrhage and isolated focal tubular necrosis. There exists a great similarity of the morphological lesions observed in the skin in the phenomenon of local skin reactivity and in the kidneys. The results of recent work of Apitz (14) suggested an almost complete parallelism between the two when the following factors in each reaction were compared; *i.e.*, regularity of occurrence; susceptibility of individual rabbits;

time of appearance of lesions; necessary interval between the preparatory and provocative injections; activity of preparations from various microorganisms; and susceptibility of animals other than rabbits.

Gerber (15) confirmed Apitz's findings in experiments with measured doses of bacterial filtrates. He found that a single injection, even though greater than the sum of two injections, given 24 hours apart, always failed to produce renal lesions. The necessary interval between the two injections was 24 hours. When the interval decreased to 6 hours, or increased to 48 hours, no lesions were obtained. However, when testicular extract was given simultaneously with the first dose it was possible to elicit the phenomenon in the kidneys within an interval of 1 or 5 hours between the injections (Bernheim and Gerber (16)). No doubt the same mechanism, namely, enhanced vascular permeability was operative in both this and the ear experiments.

It is thus evident that the phenomenon may be obtained by preparing the tissue by way of the general circulation. Thus far it has been possible to prepare the kidney only. This may be accounted for by the naturally high degree of permeability of this organ.

The state of reactivity of the phenomenon summarized in Table VI is clearly induced only by means of certain bacterial filtrates. In method 14 (Table VI), rabbits were sensitized by a single intravenous injection of horse serum 6 days prior to the experiments. In these rabbits there appeared hemorrhagic and necrotic reactions in sites of intradermal tests with horse serum when the tests were preceded by 1 hour or followed 18 to 24 hours later by an intravenous injection of potent bacterial filtrate. From these experiments it may appear that the interaction of the animal protein antigen with the circulating antibodies is capable of inducing a state of reactivity whilst the injection of bacterial filtrate provokes the reaction. The evidence brought out in this paper indicates clearly, however, that the ability of the bacterial filtrates to elicit reactions in the antigen-injected sites in sensitized animals is strictly conditioned by their skin-preparatory potency and seems to be quite independent of their provocative potency. Moreover, combinations of animal protein antigens with homologous antisera, which are endowed with provocative potency but totally devoid of skin-preparatory potencies, fail to elicit reactions in antigen-injected sites. It may be therefore assumed that in the above experiments the bacterial preparatory factors introduced into the general circulation localize at the antigen-injected sites. The possibility of localization of

substances from the blood stream at inflamed sites was clearly shown in the work of Opie (17) and his coworkers. It may be further admitted that the bacterial factors thus localized elicit the state of reactivity necessary for the phenomenon under consideration. As pointed out before, the incubation period necessary for elicitation of reactivity by way of the vascular route may be very short. The reactive sites may then, in turn, react characteristically to the toxic factors resulting from the interaction of the animal protein antigens with homologous antibodies. In this modification the shortness of the incubation period required allows a rapid succession of the phase of elicitation of reactivity and a phase of production of the lesion. The important point of the mechanism described here lies in the fact that a certain state of reactivity defined by the phenomenon under consideration is elicited subsequently to the localization of potent bacterial filtrates from general circulation, the localization by itself by no means being capable of inducing the changes described.

There is reported in the literature a number of observations dealing with so called hetero-allergic reactions in man and animals infected with live microorganisms.

Thus, Dienes (18) describes severe hemorrhagic reactions in tuberculin-injected skin sites of tuberculous guinea pigs following a shocking injection with egg white. According to Schmidt and Kraus (19), and de Potter (20), tuberculous children show general and focal reactions to injections of milk, horse serum, and broth. Similarly, a number of cases of Arthus phenomenon of extreme severity in man is recorded by Koehler and Heilmann (21), Gatewood and Baldridge (22), Irish and Reynolds (23), Tumpeer and Cope (24), Ross (25), and Maroney (26). The reactions observed consisted of severe and extensive necrosis sometimes involving large surfaces. It is of special interest that in all these cases the patients suffered from some incidental acute infectious disease (*i.e.*, diphtheria, upper respiratory infection, etc.).

Bordet (27) has already made interesting studies on the relation of these observations to the phenomenon of local skin reactivity to bacterial filtrates. An additional opportunity for the explanation of the facts is also afforded by the mechanism of elicitation of the state of reactivity, subsequently to localization of potent bacterial factors circulating in the blood stream in sites of allergic and non-specific inflammation described in this paper.

The experiments embodied in this paper seem to indicate possible concerted injury-producing effects of certain bacterial principles and nonrelated anaphylactic processes.

SUMMARY AND CONCLUSIONS

Rabbits were sensitized by a single intravenous injection of horse serum 6 days prior to the experiments. In these rabbits there appeared hemorrhagic and necrotic reactions in sites of intradermal tests with horse serum when the tests were preceded by 1 hour, or followed 18 to 24 hours later by an intravenous injection of potent bacterial filtrates. The skin-preparatory and reacting potencies of the filtrates were titrated by means of the phenomenon of local skin reactivity to bacterial filtrate. The experiments demonstrated that the ability of bacterial filtrates to elicit reactions in the antigen-injected sites in sensitized animals is strictly conditioned by their skin-preparatory potency and seems to be quite independent of their provocative potency. Combinations of animal protein antigens with homologous antisera, which are endowed with provocative potency but are totally devoid of skin-preparatory potency, fail to elicit reactions in antigen-injected sites.

The various methods of elicitation of the phenomenon of local tissue reactivity are summarized and discussed in the text.

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A SKIN TEST FOR DETECTING GROUP C HEMOLYTIC
STREPTOCOCCAL INFECTION CAUSING EPIZOOTIC
LYMPHADENITIS IN GUINEA PIGS
APPLICATIONS IN SELECTING BREEDING STOCK

By JOHANNES K. MOEN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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Epizootic lymphadenitis of guinea pigs was first reported by Boxmeyer (1). The disease is characterized by large buboes involving any of the peripheral lymph nodes, but most frequently those of the cervical chain. The lymph node enlargement is usually so marked that the involved glands frequently reach several centimeters in diameter. They are filled with thick yellowish white pus from which, almost invariably, pure cultures of hemolytic streptococci can be isolated. The spontaneous disease may be widespread in an infected herd; Boxmeyer found over 50 per cent of the animals diseased in a group of 3,000 guinea pigs. The naturally acquired disease usually runs a chronic benign course. The abscessed nodes may rupture spontaneously, drain their contents and heal. In other animals, the huge nodes cause death indirectly by mechanical pressure which interferes with vital functions. All strains of hemolytic streptococci isolated from this spontaneous guinea pig lymphadenitis fall into group C of Lancefield's serological classification (2).

During the course of tissue culture experiments on bacterial hypersensitivity induced infections with group C hemolytic streptococci in guinea pigs were studied (3). The strain of hemolytic streptococcus used was K104, originally isolated from an epidemic of spontaneous guinea pig lymphadenitis by Hardenbergh (4). The infection, induced by subcutaneous injection of broth culture, was characterized by a chronic course with the development of local abscesses, regional and distant lymphadenopathy and occasionally internal visceral involvement.

A bacterial extract, made as described below, was used in a study of cutaneous hyperreactivity, and for testing tissue cultures. The skin test dose of 0.1 cc. was injected intradermally into the shaved abdominal surface. Normal animals failed to react to this dose of bacterial extract. Infected animals, on the other hand, gave definite cutaneous reactions in all instances. A delayed inflammatory skin response similar to a positive tuberculin reaction occurred, and was characterized by redness, swelling, edema, induration and in many instances by areas of central necrosis. The skin reactions reached their height at about 24 hours and then gradually decreased in intensity over a period of several days. Positive reactions were at least 10 mm. in diameter, and many were 30 to 40 mm. in diameter with variable thicknesses and occasional areas of central necrosis. A persistent scar resulted when such necrosis occurred. Reactions were noted as early as 5 days after infection, persisted throughout the course of the disease, and for variable lengths of time in instances where apparent recovery had taken place. The intensity of the cutaneous hyperreactivity varied, depending to a certain extent on the stage of the infection. The larger and more intense reactions occurred during the acute phase, while in the chronic stage, reactions of moderate intensity were obtained.

Conditions other than active infection may induce skin hypersensitivity. Guinea pigs inoculated with repeated large doses of formolized strain K104 hemolytic streptococcal vaccines, reacted to intradermal injection of the extract. Repeated skin testing in an originally negative reactor resulted in cutaneous hyperreactivity to subsequent skin tests with the same extract. Since the bacterial extract is a crude product containing many different chemical fractions, it seems that animals infected with closely related strains or those having similar chemical components might give positive skin reactions. In a preceding study (3) it was noted that guinea pigs with induced group C hemolytic streptococcal infection gave positive skin reactions when tested with crude bacterial extracts prepared from a group B hemolytic streptococcus and from a *Streptococcus viridans* of guinea pig origin. The reactions, however, were not as intense as those obtained with the extract prepared from the homologous streptococcus.

Using the data obtained by our study (3) of the skin reactivity of infected and normal animals to bacterial extract prepared from group C

hemolytic streptococcus, the following conclusions seemed justified: first, all animals infected with group C hemolytic streptococci give a positive cutaneous reaction to a bacterial extract prepared from microorganisms of this group; second, animals recently recovered from streptococcal infection give positive reactions, but of diminished intensity; third, animals not reacting to the intracutaneous dose of streptococcal extract are free of infection with the group C hemolytic streptococcus.

The prevalence of epizootic lymphadenitis of guinea pigs is apparently widespread since infected animals were frequently obtained from various commercial dealers. The Rockefeller Institute guinea pig breeding stock also had variable numbers of infected animals. Although obviously diseased animals with large peripheral nodes were removed from the stock, this did not control the spread of the infection. It seemed possible that animals with very small lesions or undetectable internal lesions might act as reservoirs of the etiological agent and thus spread the infection. Such supposedly normal animals were found to react positively to the skin test with bacterial extract, and on autopsy deep seated lesions containing group C hemolytic streptococci were discovered. This skin test therefore showed its value in detecting disease of the internal viscera due to group C hemolytic streptococci when external evidences of the disease were lacking.

In an effort to obtain a guinea pig breeding stock free of this particular streptococcal infection it was planned to test the cutaneous reactivity with streptococcal extract and discard the positive reactors. It was presumed that a negative reactor would be free of this infection.

Preparation of Group C Hemolytic Streptococcal Extract for Skin Testing

Strain K104 streptococcus grew well in neopeptone broth. Several liters of 18 hour cultures were thrown down by centrifugation; the packed organisms were washed by resuspending in normal saline, and again centrifuged. The packed bacteria were then frozen and dried by the method of Swift (5) and ground in a ball mill for 2 or 3 weeks to disrupt completely the cells. The resulting ground bacteria were stored, and solutions of bacterial extract were prepared as needed. Since our bacterial extract was also tested in tissue culture experiments, a highly buffered saline solution (Tyrode's) was used. A solution containing 5.0 mg. of dried powdered bacteria per cc. was prepared by grinding in a mortar and by adding small amounts of Tyrode's until the required strength was reached. This solution was then centrifuged at 2,000 revolutions per minute for 45 to 60 minutes

to throw down the insoluble portion. The resulting clear, slightly opalescent solution was the bacterial extract used for skin testing. Each skin test dose of 0.1 cc. contained the soluble products of 0.5 mg. of the dried, powdered streptococci.

Selection of Animals for Breeding Stock

In January, 1935, guinea pigs were obtained from several commercial dealers. All animals were carefully inspected and palpated for lymph node enlargement, and any showing these lesions were discarded. 330 guinea pigs, 276 females and 54 males, free from external signs of streptococcal lymphadenitis, and isolated for several days were then skin tested with 0.1 cc. of bacterial extract. The skin reactions were read at 24 and 48 hours. Reactions greater than 10 mm. in diameter were considered positive. 20 positive reactors were found. The largest reaction was 33 x 18 x 2 mm. with moderate induration. The average diameter of the 20 reactions was about 20 mm. The positively reacting guinea pigs were autopsied. None showed macroscopic lesions suggestive of the hemolytic streptococcal type of infection. Several animals had areas of pulmonary consolidation from which pneumococci were cultured. 171 females and 24 males of the 310 negatively reacting guinea pigs were used for breeding purposes. They were transferred to sterilized pens in a thoroughly cleaned and disinfected room. Isolation technique was observed by the attendants. No new outside animals were added to the breeding stock.

During the 15 months from January, 1935, to April, 1936, 1,296 progeny resulted from the original negatively reacting breeding stock. No instance of hemolytic streptococcal lymphadenitis was observed in the original stock or their progeny. In April, 1936, 100 of the progeny were skin tested with a dose of streptococcal extract similar to that used originally. No positive reactions resulted.

DISCUSSION

Epizootic lymphadenitis of guinea pigs caused by hemolytic streptococcus (group C—Lancefield) is a frequent epidemic or endemic infection in herds of guinea pigs. Due to the chronic, relatively benign course of the disease, it has been difficult to eradicate the infection from large herds. Theobald Smith (6) studied the spontaneous disease in a small herd of guinea pigs in which the infection was spread by contact for a period of 9 years.

Infected animals with large peripheral nodes are readily recognized and easily eliminated. Animals with smaller foci of infection or with lesions in internal nodes or in their viscera are difficult of detection by ordinary observation, since these animals maintain good nutrition and

present no outward evidence of disease. They may, however, act as reservoirs or carriers of the infective agent, and thus maintain the infection in a herd.

The use of a group C hemolytic streptococcal extract for skin testing supposedly normal guinea pigs, and the separation of negative from positive reactors, has made it possible to obtain a breeding stock presumably free from the hemolytic streptococcus causing epizootic lymphadenitis. This presumption was strengthened by the fact that the negatively reacting breeding stock and their progeny, kept in isolation, have remained free of spontaneous lymphadenitis for a period of 15 months. Furthermore, a group of 100 of the progeny skin tested with bacterial extract gave entirely negative reactions.

The portion of the crude bacterial extract which induces cutaneous reactions is apparently stable since the dried powdered bacteria and the solutions made from them have been kept for months without losing their capacity of inducing reactions in animals known to be diseased. Caution should be emphasized in interpreting results of repeated skin tests in the same animal, since a negative reactor to the first test may eventually develop a positive reaction on repeated subsequent testings.

Skin testing with a bacterial extract prepared from a group C hemolytic streptococcus offers a method of detecting guinea pig hemolytic streptococcal carriers which show no obvious external evidence of this infection. Negative reactors are apparently free from infection with the etiological agent of epizootic lymphadenitis and may be used as a breeding stock for obtaining guinea pigs free from this infection.

SUMMARY

1. A skin test with a crude bacterial extract prepared from group C (Lancefield) hemolytic streptococci was used as a means of detecting possible carriers of the streptococcus causing epizootic lymphadenitis in guinea pigs. A positive test similar to a positive tuberculin reaction was considered presumptive evidence of present or recent infection with this streptococcus.

2. 20 positive reactors were found in 330 supposedly normal guinea pigs.

3. 195 negatively reacting animals were used as a breeding stock which yielded 1,296 progeny over a period of 15 months. None of the breeding stock or their progeny showed evidence of spontaneous lymphadenitis. Skin tests of 100 of the progeny were all negative.

4. The use of this skin test as a means of obtaining guinea pig breeding stock free of the streptococcus causing spontaneous lymphadenitis is suggested.

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THE SPECIFIC POLYSACCHARIDES OF TYPES I, II, AND III PNEUMOCOCCUS*

A REVISION OF METHODS AND DATA

BY MICHAEL HEIDELBERGER, PH.D., FORREST E. KENDALL, PH.D., AND
HENRY W. SCHERP, PH.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

(Received for publication, June 19, 1936)

For some years it has become increasingly evident that the methods originally proposed (1) for the isolation of the soluble specific substance (S) of pneumococcus were in need of revision. Avery and Goebel (2) and Enders and Pappenheimer (3) showed that more cautious manipulation of Type I pneumococcus or its culture filtrate led to the isolation of a product chemically and immunologically different from S I as originally described. Avery and Goebel presented evidence that these differences were due to a labile acetyl group which was removed by the treatment with alkali called for in the original method (1). We therefore undertook the preparation of the specific polysaccharides of Types II and III pneumococcus (S II and S III) without the use of alkali, and found no chemical differences (4) from the preparations made as originally described.

Avery and Goebel (2) had also shown qualitatively that the acetyl S I precipitated antibody from Type I antipneumococcus horse serum which the alkali-treated, deacetylated S I failed to throw down. Our quantitative determinations on a Type I antibody solution showed that the new product precipitated 2.0 mg. of antibody nitrogen as compared with 1.3 mg. thrown down by the deacetylated product. However, the S II and S III preparations which had not been alkali-treated failed to precipitate more antibody from homologous antipneumococcus horse serum than did the older preparations.

At this point we extended our quantitative study of the precipitin

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reaction between S III and homologous antibody (5) to rabbit antisera, and at once found that S III preparations which appeared identical in chemical properties, including reactivity with horse antisera, precipitated widely differing amounts of antibody from a given homologous rabbit serum (4). Similar results were obtained with S II. This was in accord with Enders and Pappenheimer's observation (3), that Type I antipneumococcus rabbit sera showed greater differences with S I prepared in various ways than did homologous horse sera.

In a search for the reason for these differences methods have been worked out for the isolation of the specific polysaccharides from culture filtrates without the use of heat, strong acid, or alkali. In general the procedure consists of the concentration of the culture filtrate *in vacuo* to a convenient volume; separation of the polysaccharide from salts and protein degradation products by repeated precipitation with alcohol in the presence of sodium acetate and acetic acid; removal of proteins by denaturation with chloroform and butyl alcohol (a modification of the method used by Sevag (6)); and elimination of any glycogen or starch present by methods depending upon the properties of the individual polysaccharides. The products were isolated as the neutral sodium salts. These were obtained entirely devoid of color and yielded solutions characterized by extraordinarily high viscosity.

EXPERIMENTAL

Since it was found necessary to vary the procedure slightly with different preparations, the following directions are given as a general guide rather than as an exact formula to be followed. The quantity of alcohol needed to precipitate the polysaccharides completely and the number of precipitations necessary to free the polysaccharides from impurities also depended to some extent upon the amount of polysaccharide present and the character of the impurities.

1. *Preparation of the Specific Polysaccharide of Type I Pneumococcus, Preparation S 120.*—10 liters of phosphate meat infusion broth containing 0.3 per cent of added glucose were seeded with a highly virulent Type I pneumococcus, strain 230.¹ After 72 hours at 37°C., 1 per cent of phenol was added and the culture

¹ Recent mouse passage is necessary for good yields of the polysaccharide. After a period of 3 weeks between the last mouse passage and the preparation of the polysaccharide the yield was reduced 80 per cent.

allowed to stand overnight. It was then centrifuged in a Sharples centrifuge and the effluent concentrated to 1 liter under reduced pressure, keeping the temperature below 35°. 100 gm. of crystalline sodium acetate were dissolved in the concentrated broth and 1250 ml. of 95 per cent alcohol were added with constant stirring. The polysaccharide separated as a white curdy mass which settled rapidly. After standing overnight the supernatant was poured off and the precipitate centrifuged. Usually a three layer separation was obtained as described (1), but in cases in which only a single volume of alcohol was used the phosphates and other salts were not thrown out and no syrupy layer was found. The three layer separation was also not obtained in the absence of phosphates. If the broth used is phosphate-free, purification of the polysaccharides may often be greatly facilitated by addition of sufficient phosphate to bring about the three layer separation. A solution of 10 gm. of sodium acetate in 250 ml. of water was made acid to litmus with acetic acid and the alcohol precipitate (or middle layer) was dissolved in this and reprecipitated with 300 ml. of 95 per cent alcohol. The centrifuged precipitate was dissolved in 250 ml. of water containing 10 gm. of sodium acetate and 5 ml. of glacial acetic acid, and the turbid solution was shaken with 50 ml. of chloroform and 10 ml. of *n*-butyl alcohol for 30 minutes (*cf.* 6). On centrifugation a semisolid emulsion separated between the aqueous layer and the chloroform. The solution and the chloroform were poured off and the layer of emulsion was washed with two 50 ml. portions of water which were saved for washing later emulsion layers. 50 ml. of chloroform and 10 ml. of butyl alcohol were added to the aqueous layer and the shaking was repeated. On centrifugation a smaller semisolid emulsion layer was formed and this was treated as before. Shaking with chloroform was repeated as long as an emulsion layer formed. Seven shakings were usually required. The washings of the emulsion layers were combined and shaken with fresh additions of chloroform as long as an emulsion layer formed, and were then combined with the main aqueous solution, the total volume now being 350 ml. The polysaccharide was precipitated with 500 ml. of 95 per cent alcohol, redissolved in 250 ml. of water, and the solution tested for phosphate and glycogen. Phosphate may be removed either by repeated precipitations with alcohol in the presence of sodium acetate and acetic acid or with glacial acetic acid in the presence of sodium acetate. Glycogen may be left behind by precipitating the S I from a salt-free aqueous solution with copper acetate. In the present instance the solution was free from phosphate but gave a strong iodine test for glycogen. 20 ml. of a saturated solution of copper acetate slightly acidified with acetic acid were added and the resulting bluish precipitate was centrifuged off. The supernatant remained clear when more copper acetate was added. The precipitate was dissolved in 50 ml. of 20 per cent sodium acetate solution and 5 ml. of glacial acetic acid, and reprecipitated with an equal volume of alcohol. This was repeated until the precipitate was free from copper, after which it was dissolved in 100 ml. of water and the solution again tested for glycogen. If this is present the copper precipitation is repeated. The S I was finally precipitated with redistilled alcohol in the presence of a small

amount of sodium acetate, washed with redistilled alcohol, filtered, and dried. Yield: 0.9 gm. of the neutral sodium salt of S I.

2. *Preparation of the Specific Polysaccharide of Type II Pneumococcus, Preparation S 84.*—10 liters of a 4 day culture of Type II pneumococcus, strain B 39, were treated in the same way as for Type I. No differences were observed up to the point at which the copper acetate precipitation was made. However, only one-third of the S II was thrown down as the copper salt. This fraction, S 84 A, which was free from glycogen, was reprecipitated as the copper salt and isolated separately.

The copper-soluble fraction, S 84 B, was freed from copper salts by several precipitations with alcohol in the presence of acetic acid and sodium acetate and was redissolved in a small volume of water. Even this concentrated solution failed to react with copper acetate. As glycogen was present in this fraction it was removed by adjusting the pH to 6.5 and adding a small amount of saliva. After a few minutes the iodine test was negative. To remove protein impurities added in the saliva 5 gm. of sodium acetate and 2.5 ml. of acetic acid were added, and the solution (volume 100 ml.) was repeatedly shaken with chloroform and a little butyl alcohol until an emulsion layer was no longer formed. Since the polysaccharide solution still contained nitrogen it was adjusted to contain 5 gm. of sodium acetate per 100 ml. and was chilled and precipitated with 5 volumes of glacial acetic acid. The precipitate was centrifuged in the cold, taken up in 50 ml. of 5 per cent sodium acetate solution, and again precipitated with 5 volumes of acetic acid. This was followed by two precipitations with alcohol from 5 per cent sodium acetate solution and one precipitation with redistilled alcohol, after which the polysaccharide was washed with redistilled alcohol, filtered, and dried.

	gm.
Yield of sodium salt of copper-precipitable S II.	0.163
“ “ “ “ “ copper-nonprecipitable S II.	0.302
Total.	0.465

The S II content in the original broth was determined according to Reference 7. 1 ml. of the broth contained 0.59 mg. or a total of 0.590 gm. of S II in the 10 liters used. Recovery, 78 per cent.

Preparation S 85.—9 liters of an 18 hour culture of Type II pneumococcus were worked up as above except as follows: Glycogen was removed with saliva after the first shaking with chloroform. The proportion of copper-precipitable S II, S 85 A, was much greater in this lot. Of 406 mg. of S II present in the broth 300 mg. were isolated from the copper-precipitable fraction and 60 mg. from the nonprecipitable fraction. The latter portion was not rigorously purified.

3. *Preparation of the Specific Polysaccharide of Type III Pneumococcus.*—A number of procedures for the isolation of S III were tried before the simplified method given in the following paragraph was found. Thus in preparation S

105 B, the culture filtrate was concentrated *in vacuo* to 1/10 its original volume. After three precipitations with 1.5 volumes of alcohol, part of the protein contained in the material was removed by 40 per cent saturation with sodium sulfate at 37°. The S III was then precipitated by completely saturating the solution with sodium sulfate. After removing the sodium sulfate by repeated precipitations of the S III with alcohol and acetic acid in the presence of sodium acetate the product was found to contain 2.4 per cent of nitrogen. This was removed by twice precipitating the S III as the barium salt with barium chloride. The barium was removed by repeated precipitations from 20 per cent sodium acetate solution with acetic acid and alcohol. The S III was isolated as the neutral sodium salt.

Similar methods were used in preparation S 107 with the omission of the sodium sulfate precipitations.

Preparation S 108.—9 liters of a 4 day culture of Type III pneumococcus, strain A 66, were worked up in the same way as the Type I culture. Only minor differences in behavior were noted. Thus, in the presence of sodium acetate less alcohol was needed to precipitate this polysaccharide, 1 volume of alcohol instead of 1.25 volumes being sufficient. Instead of giving a test for glycogen this preparation showed a distinct blue color when tested with iodine. This starch-like substance remained in the supernatant when the S III was precipitated with copper. Yield of the neutral sodium salt of S III: 1.08 gm.

4. Chemical and Physical Properties of Specific Polysaccharides of Types I, II, and III Pneumococcus.—The chemical and physical properties of different preparations are summarized in Table I. In this table the preparation designated S I old was prepared by the short method as described in an earlier paper (8); S 91 A by concentrating the culture filtrate on the steam bath and isolating the polysaccharide without the use of alkali or strong acid; S 120 as described above. Of the Type II preparations, S II old was prepared by the original method (1) but was fractionated by precipitation with copper; S 80 A was obtained from heated broth without the use of strong acid or alkali, and S 83 E, S 84 A, S 84 B, and S 85 A from broth concentrated *in vacuo* and isolated without the use of strong acid or alkali. Of the Type III preparations, S III old and A 66 were prepared as in Reference 1, S 102 from broth concentrated on the steam bath and isolated without the use of strong acid or alkali; S 105, S 107, and S 108 by methods described in this paper.

Analyses for nitrogen were made by a modified micro Kjeldahl method. The acetyl content was determined by hydrolyzing with 25 per cent *p*-toluenesulfonic acid solution, to which a small amount of barium hydroxide had been added, in an all glass reflux apparatus heated in a bath of boiling saturated sodium chloride solution for 2 to 4 hours. The acetic acid formed was distilled in a current of steam in a micro Kjeldahl apparatus. Successive 100 ml. samples of the distillate were collected, heated to boiling under reflux, and then cooled in an ice bath in a current of carbon dioxide-free air. They were then titrated with $N/70$ sodium hydroxide using phenolphthalein as indicator. In general all of the

acetic acid was contained in the first two fractions. The uronic anhydride was determined by the method of Burkhart, Baur, and Link (10) and reducing sugars, after acid hydrolysis, by the Hagedorn-Jensen method (11). Viscosities were measured in 0.9 per cent salt solution with an Ostwald viscometer at 20°C. As the values for the viscosity in water are greatly affected by the presence of small amounts of salt they are less useful for comparing different preparations than are the values in salt solutions.

In Table II are summarized data on the maximum amount of antibody nitrogen specifically precipitable by the different polysaccharide preparations from homologous antisera produced in the rabbit and in the horse. In order to remove antibodies that might react with material from the culture other than the polysaccharide (12) all of the antisera were first completely absorbed with somatic carbohydrate "C" (13) derived from heterologous strains of pneumococcus, and with pneumococcus protein obtained from an R strain. As described in previous papers (5), a slight excess of the polysaccharide was mixed with 1.0 ml. of the serum or antibody solution at 0° in a total volume of 4 ml., and the quantity of antibody nitrogen in the washed precipitate was determined by the micro Kjeldahl method. Rabbit sera were allowed to stand for 48 hours in the refrigerator after mixing with the polysaccharide, and the horse sera for 24 hours to insure completion of the reaction.

5. Effect of Various Procedures on Reactivity of Polysaccharides with Rabbit Antisera.—In order to determine which of the preparative manipulations used in the original isolation of the polysaccharides were responsible for the lowered reactivity of the older preparations with rabbit antisera, the following experiments were performed.

Type I. Effect of Heat.—Solutions of S 120 containing 1.0 mg. per ml. in 0.9 per cent NaCl and 2.0 mg. per ml. in water at pH 6.4 were sealed in glass tubes and heated for 6 hours in a boiling water bath. The relative viscosity of the solution in salt fell from 1.69 to 1.05 while the viscosity of the solution in water fell from 9.0 to 1.22. 12.4 ml. of the heated aqueous solution containing 24.8 mg. of S 120 were precipitated with 90 ml. of alcohol with the addition of a few drops of sodium sulfate solution to assist flocculation. The precipitated polysaccharide was centrifuged off and the supernatant concentrated to dryness on the steam bath after the addition of 5 ml. of 10 per cent sodium hydroxide solution to prevent loss of acetic acid. The residue was taken up in water, acidified with sulfuric acid, and the volatile acids were determined. Blank analyses were made on the reagents used. Volatile acids equivalent to 0.44 mg. of acetyl group were found, or 25 per cent of the acetyl content of the original polysaccharide. Part of the heated polysaccharide was precipitated when the pH of the solution was brought to 3.6 with acetic acid (*cf.* 1).

Effect of Alkali.—To 12.5 ml. of solution containing 50 mg. of S 120 were added 3.13 ml. of 10 per cent sodium hydroxide solution to make the concentration of alkali 0.5 N. The solution was allowed to stand in an incubator at 37°C. for 48 hours and the alkali was neutralized with hydrochloric acid to pH 7.0

and the volume made up to 50 ml. The final concentration of sodium chloride was 0.91 per cent and the relative viscosity of the solution was 1.20. 10 ml. of

TABLE II

Maximum Antibody Nitrogen Precipitated at 0°C. by Pneumococcus Polysaccharides from Homologous Antisera

Type I						
Preparation.....	S I old	S 91 A	S 120	S 120 heated	S 120 treated with alkali	S 120 heated and treated with alkali
Serum						
	mg.	mg.	mg.	mg.	mg.	mg.
Rabbit 3.70.....	0.08	0.21	0.48	0.26	0.14	0.07
Rabbit 3.41.....	0.15	0.26	0.51			
Horse antibody solution B 77.....	0.62	0.71	0.75	0.72	0.65	

Type II						
Preparation.....	S II old	S 80 A	S 83 A	S 84 A	S 84 B	S 85 A
Serum						
	mg.	mg.	mg.	mg.	mg.	mg.
Rabbit RS 1.....	0.70	0.70	0.97	0.98	0.99	1.01
Horse antibody solution B 83.....	0.72	0.73		0.71	0.71	

Type III										
Preparation.....	S III old	A 66	S 102	S 105	S 107	S 108	S 108 acid	S 108 acid, heated in air	S 108 acid + broth	S 108 acid + broth, heated in air
Serum										
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Rabbit antibody solution B 53.....	0.33	0.62	0.56	0.78						
Rabbit serum 3.50.....	0.86	1.12	1.10	1.25*	1.18†	1.27	1.23			
Rabbit serum 3.51.....	0.94	1.24	1.05			1.45	1.44	1.36	1.43	1.24
Horse serum 607.....	1.34	1.34	1.32	1.37	1.37					

* S 105 after treatment with N/2 NaOH at room temperature for 24 hours precipitated 1.22 mg. antibody N from this serum.

† S 107 after heating 6 hours at 100° in a sealed tube precipitated 1.17 mg. antibody N from this serum.

this solution were sealed in a glass tube and heated in a boiling water bath for 6 hours.

Data on the reaction with antisera of the polysaccharide treated as described above are included in Table II.

Type II.—A solution of preparation S 83 E in phosphate buffer at pH 5.8 was heated in a small evaporating dish in a boiling water bath for 8 hours, keeping the volume constant by addition of water. A similar solution was sealed in a glass tube *in vacuo* and heated in a boiling water bath. Samples of the culture filtrate were treated in the same way. After cooling, the solutions were neutralized to pH 7.0 with sodium hydroxide and set up in excess with 1 ml. of rabbit serum in a total volume of 11 ml. at 0°C. and the antibody nitrogen precipitated was determined after 72 hours. The large volume taken was considered desirable to avoid errors due to the high salt concentration of the neutralized broth.

Antibody Nitrogen Precipitated from Rabbit Serum RS 1

	Unheated	Heated <i>in vacuo</i>	Heated in air
	mg.	mg.	mg.
Broth.....	1.07	1.02	0.86
S 83 E.....	0.94	0.94	0.84

The difference in the amount of nitrogen precipitated by the broth and the isolated polysaccharide is believed to be due to antibodies to somatic carbohydrate and protein remaining in the serum in spite of the preliminary absorption. Quantitative agglutinin determinations (12) showed that there was still 0.17 mg. of non-type specific antibody nitrogen in the serum removable with pneumococcus I R suspension. The viscosities of solutions of S 85 A containing 1 mg. per. ml. in 0.9 per cent sodium chloride solution were compared after heating at 100°C. in sealed tubes at pH 6.6 for 6 hours, after heating in a current of air for 6 hours, and after treating with N/2 sodium hydroxide solution at 37° for 24 hours. The maximum amount of nitrogen precipitated from rabbit serum RS 1 was also determined.

	S 85 A	S 85 A heated in sealed tube	S 85 A heated in current of air	S 85 A alkali-treated
Relative viscosity 1 mg. per ml. in 0.9 per cent NaCl.	1.64	1.34	1.21	1.51
Mg. antibody N pptd. from 1 ml. RS 1.	1.01	1.00	0.99	1.00

Type III.—Preparation S 105 was treated with N/2 sodium hydroxide solution for 24 hours at room temperature without greatly changing its power to precipitate rabbit antibody (Table II). Heating preparation S 107 for 6 hours in a sealed tube at 100° reduced its viscosity from 2.64 to 1.34 without changing its reactivity. Purification of part of preparation S 108 by precipitation with

strong hydrochloric acid at 0° as described in the earlier papers (1) reduced its viscosity (S 108 acid) from 3.11 to 2.23 without affecting its ability to react with rabbit antiserum. Heating this preparation at pH 5.5 in a sealed tube at 100° also failed to change its reactivity, but heating at 100° in a current of air reduced it slightly. However, when this preparation was heated in the presence of sterile broth in a stream of air the amount of antibody precipitated from a rabbit antiserum was reduced to a still greater extent (Table II).

6. *Dialysis Experiments.*—In an earlier paper (14) it was reported that while S III would not diffuse through a collodion membrane from an aqueous solution into water, it would diffuse if the solutions both inside and out of the dialysis bag contained 10 per cent sodium chloride. The experiment has been repeated on the S III preparations described in this paper.

Collodion bags were made in test tubes with 6 per cent parlodion-ether-alcohol-acetic acid mixture (15). Aqueous solutions of preparations S III old, S 102, S 108, and S 108 heated with broth, were placed in the bags, and they were immersed in beakers of distilled water for 18 hours. At that time the solutions in the beakers were tested for the presence of polysaccharide with homologous horse and rabbit antiserum. The solutions in the bags were then replaced by solutions of S III containing 10 per cent sodium chloride and dialysis continued against 10 per cent sodium chloride solution in the beakers. S 108 was also tested in the presence of 15 and 20 per cent sodium chloride.

Reaction of Dialysate with Immune Serum

Salt concentration	0		10 per cent		15 per cent		20 per cent	
	Horse	Rabbit	Horse	Rabbit	Horse	Rabbit	Horse	Rabbit
Antiserum.....								
S III old.....	—	—	++	++				
S 102.....	—	—	++	++				
S 108.....	—	—	—	—	—	—	—	—
S 108 (heated).....	—	—	+					

7. *Additional Data on Constitution of S I.*—Work is in progress in this laboratory on the structure of the Type I pneumococcus specific polysaccharide. Analyses show that the alkali-treated partially deacetylated S I (Table I) contains 5.12 per cent of nitrogen and 65 per cent of uronic anhydride or one atom of nitrogen per molecular weight of 273 and one molecule of uronic anhydride for every 271 molecular weight.

Preparation S 120, not alkali-treated, with 4.62 per cent nitrogen, 56 per cent uronic anhydride, and 7.1 per cent acetyl contains one nitrogen for each 303, one uronic anhydride for each 314, and one acetyl group for each 605 molecular weight. In both preparations about half of the nitrogen is free amino nitrogen.

Acetylation of the deacetylated S I with acetic anhydride in the presence of sodium carbonate followed by treatment with N/2 sodium hydroxide at room

temperature gave a product which contained 4.43 per cent nitrogen, 54.7 per cent uronic anhydride, 13.2 per cent acetyl, and no free amino nitrogen. This corresponds to one nitrogen for each 316, one uronic anhydride for each 322, and one acetyl for each 326 molecular weight. In contrast to the original product and to the S 120, both of which are good buffers at the neutral point, this material titrates sharply with phenolphthalein as indicator and shows an acid equivalent of 319. This indicates that the carboxyl groups of the uronic anhydride are free and that none of the nitrogen is present as an acid amide.

Treatment of S I with nitrous acid destroys its serological activity (1) and gives a product which has one-third the reducing value of glucose as measured by the Hagedorn-Jensen (11) method.² Upon hydrolysis of S I with hydrochloric acid this same reducing value is obtained.

The data agree with the hypothesis that the basic unit of the molecule is a trisaccharide containing two molecules of uronic acid and an unidentified substance containing two atoms of nitrogen. As already indicated by the isolation of mucic acid (1) part of the uronic acid, at least, is galacturonic acid. After hydrolysis of S I with methyl alcoholic hydrochloric acid as described by Morell and Link (16) the crystalline methyl-*d*-galacturonide methyl ester monohydrate was isolated.

DISCUSSION

In the preceding portion of the paper revised methods have been given for the isolation of the specific polysaccharides of Types I, II, and III pneumococcus. The procedure is relatively simple, avoids the use of heat, alkali, or mineral acid, and results in the recovery of high yields of polysaccharide as entirely colorless sodium salts. For good yields recent animal passage of the pneumococcus strain appears essential. Since the manipulation is less drastic than any other hitherto proposed, except that of Sevag (6) which is followed in part, it is believed that the products represent the closest approach yet attained to the carbohydrates as they are given off by the pneumococcus cell to the surrounding culture medium.

The product isolated by Sevag (6) from Type I organisms contained 6.7 per cent nitrogen, 1.3 per cent amino nitrogen, and had an optical rotation $[\alpha]_D^{217}$.³ The high nitrogen, low amino nitrogen, and optical rotation indicate the presence of a nitrogen-containing component not present in our products. The question of whether this component is

² Details of this work will be given in a later communication.

³ Through a typographic error this value was given as 21.7° in Sevag's paper.

present as an impurity or is an integral part of the polysaccharide as it exists in the organisms cannot be settled at present.

While the preparations now reported may be artefacts just as were the older ones, they are certainly a step closer to the native substances themselves, and it is proposed to refer to these products as the specific polysaccharides of Types I, II, and III pneumococcus (S I, S II, S III). The distinction between acetyl S I and S I is not made, since Avery and Goebel (2) have made it obvious that S I, properly isolated, should contain the acetyl group.

An unexpected result is the finding that the specific polysaccharides of pneumococcus are not thermostable, as was thought when only qualitative methods were available for their study. As will be noted from the data in Table I the viscosity is the most sensitive indicator of the change on heating, dropping markedly without any accompanying change in reactivity with antisera (Table II) in the case of S II and S III, and with a decrease in precipitating power in the case of S I owing to the partial removal of acetyl. When air is admitted during the heating an even greater decrease in viscosity occurs, and this is accentuated in the presence of broth, resulting in a decrease in the precipitating power of all three polysaccharides. This is probably the reason for the inferior precipitating power toward rabbit antisera of the older preparations, including even the acetyl S I, since heating was a step in the isolation. From the marked diminution in viscosity it is probable that heating effects a partial depolymerization of the long, thread-like chains of native polysaccharide, for Staudinger (17) has shown a definite relation between the viscosity and chain length of polymer homologues. It would appear, however, that the depolymerization, if such it be, may proceed quite far before the ability of the specific polysaccharide to precipitate rabbit antisera is markedly affected. On depolymerization by more drastic treatment, such as partial hydrolysis by mineral acid, the precipitating power toward rabbit antisera may be entirely lost, while the amount of antibody thrown down from horse antisera merely diminishes (18). Acid cleavage is also accompanied by further decreases in viscosity (14).

In harmony with the above are the dialysis experiments with S III (page 568). It had been shown previously that S III prepared according to Reference 1 did not pass through a collodion membrane into

water, but dialyzed readily in the presence of 10 per cent salt solution (14). It has now been found that a new preparation of S III which had not been heated failed to dialyze even in the presence of strong salt, but that after heating with broth dialysis occurred.

It is believed that the simplest explanation of the above is that the unheated preparations have the largest particle size, or longest chain, and that heating results in a degradation of the molecule to smaller units.

The instability of the pneumococcus specific polysaccharides toward heat has thus been shown by three independent methods. In addition to the probable depolymerizing action of heat there is evidence that an oxidative process occurs in the presence of air, and that this is greatly enhanced in the presence of broth, under the conditions similar to those used in the older preparations (1, 2), by the catalysts present in this material (19).

For the above reasons, also, the S III reported by Hornus and Enders (20) would appear to be a degradation product and not a different substance. Possibly the added amount of antibody thrown down by the preparation is due to contamination with C substance.

Work on the constitution of the specific polysaccharide of Type I pneumococcus has been continued. Indirect evidence had previously been obtained of the presence of galacturonic acid in the molecule (1). This has now been confirmed by the actual isolation of the methyl glycoside of galacturonic methyl ester from the products of hydrolysis of S I by methyl alcoholic hydrochloric acid. The analytical data so far obtained are consistent with the assumption of a trisaccharide unit for the S I molecule, containing two molecules of uronic acid, possibly both galacturonic acid, and two atoms of nitrogen. Further work is in progress.

SUMMARY

1. The thermolability of the specific polysaccharides of Types I, II, and III pneumococcus has been shown by three independent methods: (a) diminution of the viscosity of solutions on heating; (b) decrease in the amount of antibody precipitated from homologous rabbit antisera; and (c) increased tendency (S III) to pass through a collodion membrane.

from 40 to 49 mm. = 5 plus. When the area of swelling was ill defined, one-half was deducted from the grade. Hemorrhages or necrosis did not occur in the skin reactions.

The complement fixation tests were carried out in the following way. The rabbit sera were heated for 30 minutes at 55°C. As antigen a suspension of the Ravenel strain was used. It was prepared by grinding a culture of Ravenel strain and suspending it in normal saline so that 1 cc. of the suspension contained 10 mg. of tubercle bacilli. The suspension was heated for 30 minutes at 60°C. and 0.35 per cent tricresol was added as a preservative. About one-fourth of the self-inhibition dose was used for the complement fixation. The complement was either fresh or dried guinea pig serum. It was always freshly titrated and two and a half units were used. The concentration of sheep cells suspension was 5 per cent (in relation to whole blood). Two units of hemolysin were employed. 0.25 cc. of each ingredient was incubated in a water bath at 37°C. for 1 hour. To check the accuracy of the complement fixation tests, a standard serum was always included, which was obtained from a rabbit that was immunized by a series of injections of tubercle bacilli. 0.5 cc. portions of the serum were dried *in vacuo* in the frozen state by the method of Elser, Thomas, and Steffen (1), and a sample of dried serum (recovered by adding 0.5 cc. of distilled water to the dry material) was run in the complement fixation test. That the method of drying yielded uniform samples was shown by testing three samples of dried serum simultaneously. The variation in the complement fixation test from week to week was very slight, if any, since the highest dilution of the standard serum that fixed complement was always the same.

EXPERIMENTAL

In group 1 (Charts 1 and 2) rabbits were injected with 0.000001, with 0.0000025, and with 0.00001 mg. tubercle bacilli respectively, some intravenously and some intratracheally. The rabbits died with extensive tuberculosis from 102 to 159 days after the infection. The lungs weighed from 52 to 125 gm., most of them weighing from 70 to 80 gm. (the weights of normal lungs vary from about 10 to 12 gm.) and about nine-tenths of their cut surface was involved in tuberculosis. There were many tubercles in the kidneys and in some animals the spleen, liver, and cecum contained tubercles. Two to four rabbits in each group were tested weekly with tuberculin. The first tests were made 1 week before, and second tests 1 week after the inoculation with living tubercle bacilli. It is noteworthy that the duration of life and the character of lesions were about the same in rabbits subjected to weekly tuberculin tests and in those not tested.

The progress of sensitization as indicated by the results of tuberculin tests performed weekly followed a fairly uniform course. Sensitization was evident from 2 to 4 weeks after infection. In one instance the reaction was 4½ plus, at the end of 2 weeks, but in the remaining instances from 3 plus to 4 plus at the end of 3 or 4 weeks was usual. Subsequently for a period of about 10 weeks

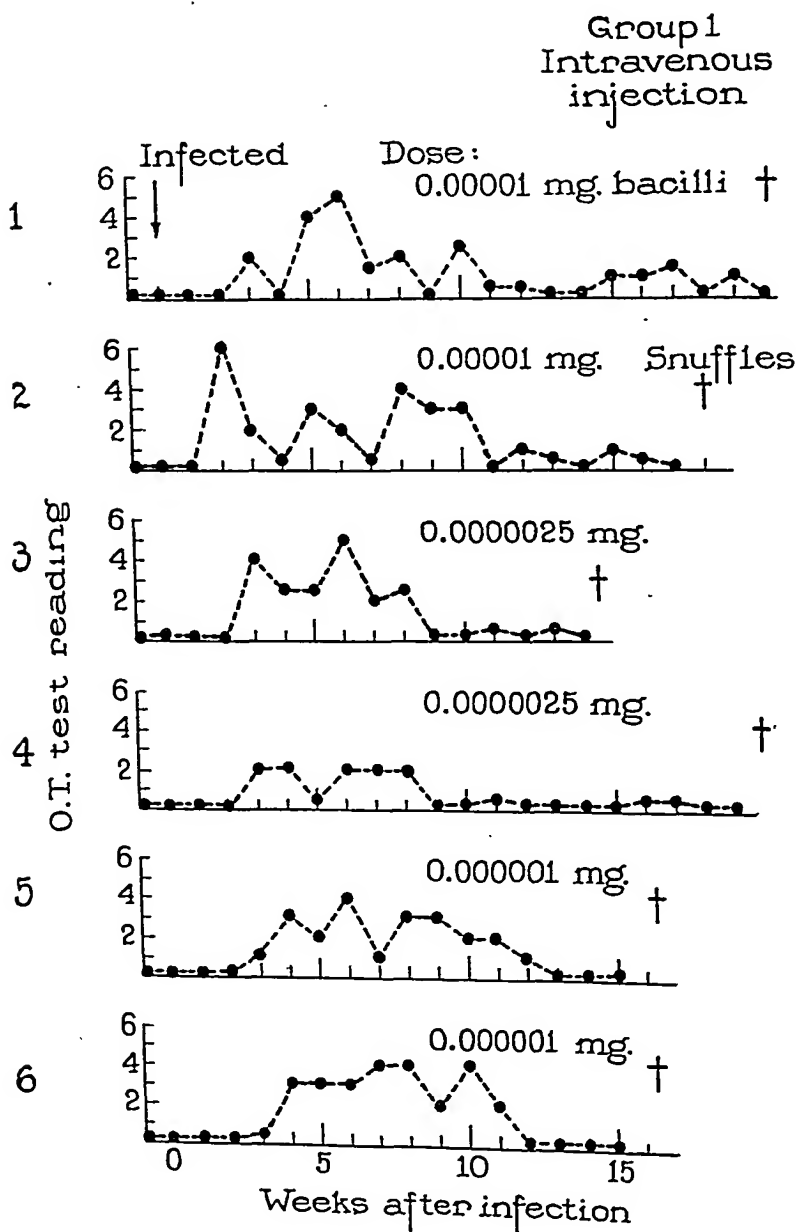


CHART 1. Old tuberculin tests.

there was a fairly intense sensitization, sometimes reaching a maximum of 4 plus or 5 plus, but with fluctuation of level. At a later period, *i.e.* from the 10th to the 15th weeks, sensitization diminished, in most instances disappeared, so that it was often almost completely absent during 5 or more weeks preceding death. In this group of rabbits serological tests were not made.

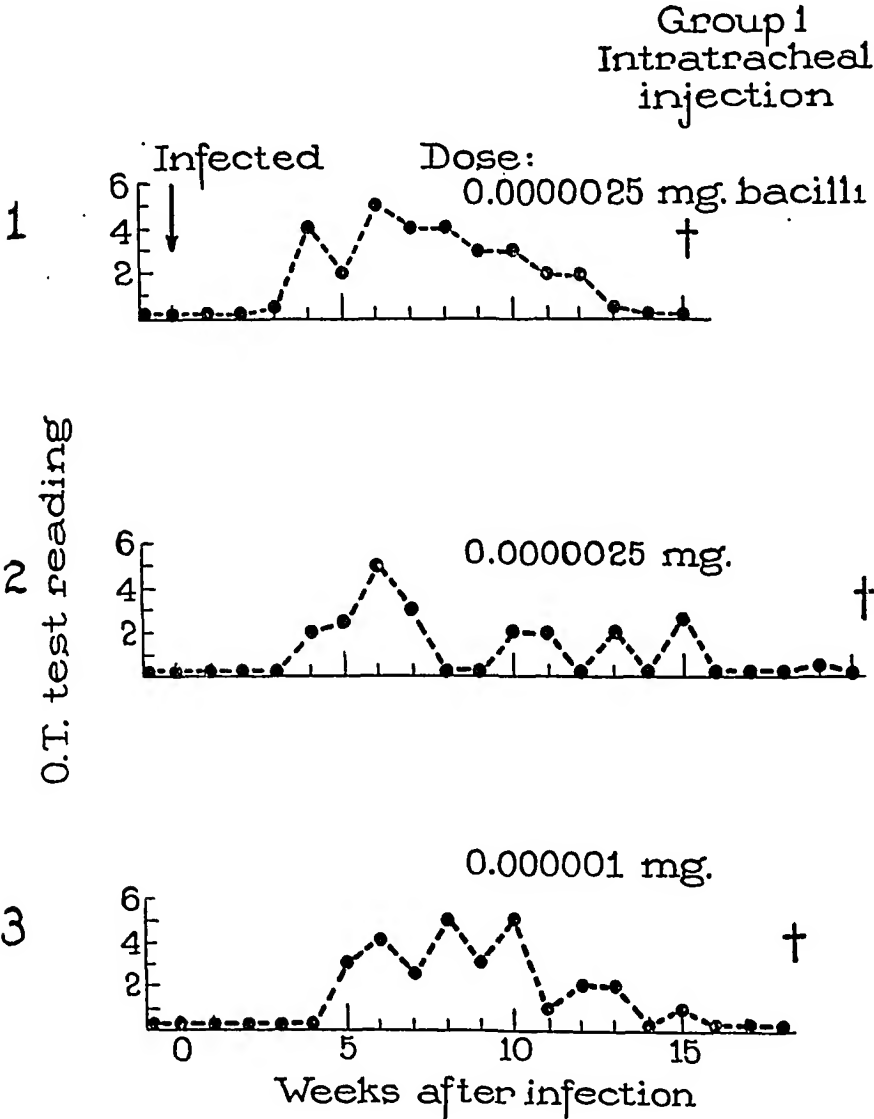


CHART 2. Old tuberculin tests.

In group 2 (Chart 3) six rabbits were infected with 0.00001 mg. tubercle bacilli and the intensity of both sensitization and antibody formation was followed at weekly intervals. The animals died after from 112 to 155 days, with extensive tuberculous lesions. The lesions found at autopsy were very similar to those in group 1. One of the rabbits, No. 1, developed snuffles during the 3rd week after infection.

Intense sensitization became evident in five of the six rabbits during the 4th or 5th week and in one during the 6th week. The sensitivity was maintained for from 3 weeks to almost 3 months. In five rabbits the intensity of the tuberculin reactions diminished gradually from week to week and in one animal rapidly (in 1 week). None of the six rabbits reacted to tuberculin for a period of 2 to 10 weeks preceding their death.

The graph that represents the antibody titers from week to week during the course of the disease is fairly uniform in five of the six rabbits and very different from the shape of the graph of the tuberculin sensitiveness. Before the infection complement fixation reaction was obtained with 1:10 dilutions of the sera in two instances, with 1:5 dilution in two instances, and the sera of the remaining two rabbits did not react in the latter dilution. Antibodies developed and were maintained for long periods in all rabbits except in the animal with snuffles. Significant titers were observed from 3 to 7 weeks after infection. There was some fluctuation of the antibody titers; however, antibodies were present in all rabbits throughout the whole course of the disease. The persistence of antibodies was in striking contrast with the disappearance of skin sensitiveness to tuberculin.

In group 3 (Chart 4) six rabbits were infected with a relatively large dose of 0.001 mg. tubercle bacilli, and as in group 2 tuberculin sensitiveness and antibody formation were followed by weekly tests. Four of these animals were killed *in extremis* from 77 to 98 days after infection, one died of tuberculosis after 110 days, and one was killed because of snuffles and diarrhea 84 days after infection. All the rabbits had extensive tuberculosis of the lungs and kidneys.

In most of the rabbits sensitization was evident at the 3rd week and lasted from 1 to 6 weeks. One of the six rabbits, No. 6, showed a fairly strong tuberculin reaction in only one of the weekly tests, 7 weeks after the infection. In all animals sensitization disappeared several weeks before they became moribund.

Complement fixing antibodies appeared during the 4th week. In all rabbits there was a rapid and conspicuous increase in antibody titer during the course of the disease. When the antibody titers reached their maximum height they remained at that level almost until the death of the animal, with slight, if any, decrease, except in one instance (rabbit 4). In this rabbit the drop of antibody titer was considerable, *i.e.* from 1:120 to 1:30.

The comparison of the curves of hypersensitiveness and antibody formation shows the following: The development of tuberculin sensitiveness as a rule preceded the formation of antibodies and the maxima of the two curves did not

Group 2

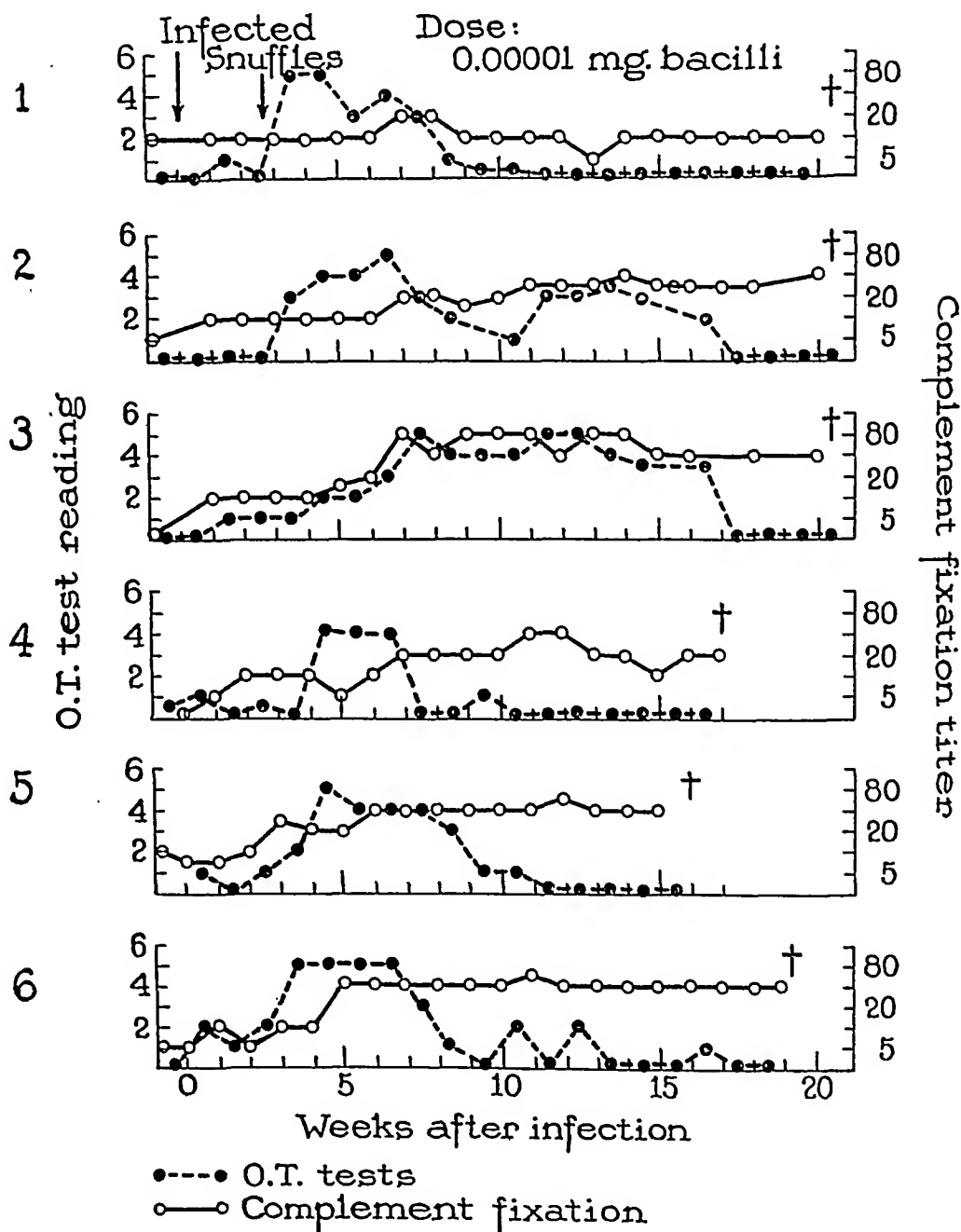


CHART 3. Old tuberculin tests and complement fixation reaction.

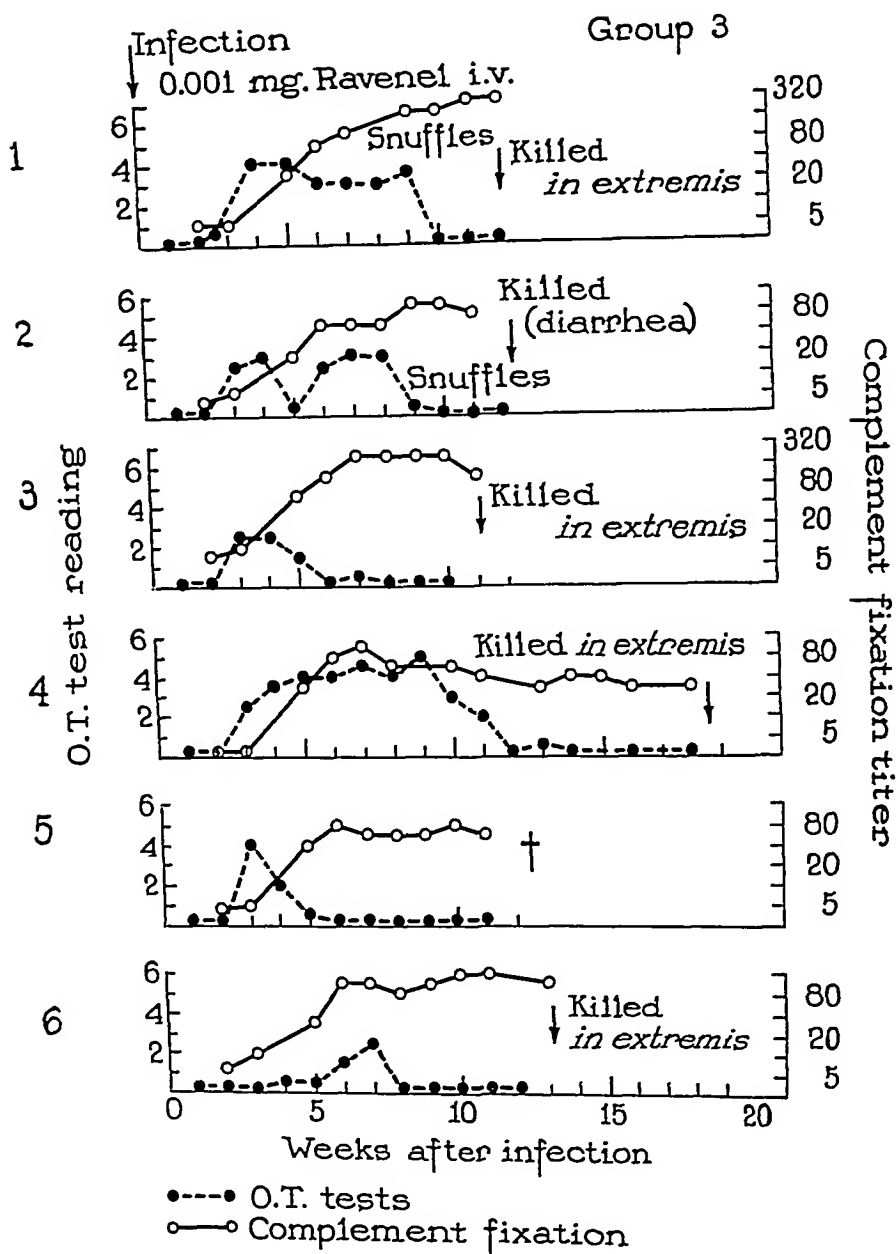


CHART 4. Old tuberculin tests and complement fixation reaction.

coincide. The tuberculin sensitiveness disappeared several weeks before death, while the antibodies persisted in the blood until the animals became moribund or died. The intensity of tuberculin reaction and antibody formation was dissimilar in the individual rabbits, although the animal (rabbit 1) that showed the most abundant antibody formation reacted most intensely.

DISCUSSION

The data presented show that all rabbits infected with various amounts of highly virulent tubercle bacilli (from 0.000001 to 0.001 mg.) developed allergic skin sensitiveness to tuberculin and antibodies demonstrable by the complement fixation test. The intensity and the duration of hypersensitiveness varied in the different animals even within the same group. In spite of this variation in all instances during the course of the disease in relation to skin reaction, three periods, a pre-allergic, an allergic, and a post-allergic period, can be recognized in all animals.

Antibody formation was evident in all rabbits that lived more than 3 weeks, save one (rabbit 1, Chart 3) that developed snuffles during the 3rd week after the injection of 0.00001 mg. of tubercle bacilli. The production of antibodies was more rapid and the titers of the sera were higher in rabbits injected with 0.001 mg. of tubercle bacilli than in rabbits that received from 0.001 to 0.000001 mg. of tubercle bacilli.

The disappearance of tuberculin sensitiveness in the presence of progressive tuberculosis is known to occur in man when miliary tuberculosis develops, and sometimes before death. Tuberculin sensitiveness often decreases or vanishes during measles or whooping cough, but it returns after recovery from these diseases. Some tuberculous guinea pigs do not react to intracutaneous injection of tuberculin for a short period of time preceding death.

An almost complete absence of the capacity of the skin to react to tuberculin was observed in young tuberculous guinea pigs, highly allergic in the systemic tuberculin test (Freund (2), Valtis (3)). The skin of tuberculous albino rats does not react to tuberculin although tuberculin death may be produced with small doses of tuberculin in some tuberculous rats (Smith (4), Freund and Hehre (5)).

In interpreting the disappearance of tuberculin skin reactions, it should be remembered that tuberculous rabbits or guinea pigs do not lose their allergic sensitiveness to the toxic effect of tuberculin in the

systemic reaction. Three of four rabbits tested 80 days after the injection of 0.0001 mg. bovine tubercle bacilli and after they had lost their skin sensitivity, died with tuberculin death following the intravenous injection of 1 cc. of tuberculin.

The question naturally arises as to the nature of the desensitization observed. The relationship of skin sensitiveness to antibodies was best studied with sensitization of rabbits to complex and single protein antigens. Opie (6) found that there is a correlation between the precipitin titers of the serum and sensitiveness in the Arthus reaction. When a complex antigen such as horse serum is used, it is not possible to desensitize rabbits completely; however, when a simple antigen, crystalline egg albumin, is used for sensitization a large amount of antigen causes a complete desensitization that is accompanied with a loss of serum precipitins. When the sensitiveness returns, antibodies reappear in the serum. The data presented in this paper show that when skin sensitiveness disappears with the progress of tuberculosis, there is no diminution of complement fixing antibodies.

In correlating the tuberculin tests with the antibody titration, it should be taken into consideration that in these two tests two different preparations were used. Tuberculin, however, acts also as antigen in complement fixation and heat-killed tubercle bacilli elicit tuberculin-like reactions in the skin of allergic rabbits. It is difficult to compare the potency of these preparations: the suspension of heat-killed tubercle bacilli and tuberculin in the two tests. Tuberculin preparations are feeble "antigens" in the complement fixation test.

CONCLUSIONS

1. Rabbits infected with bovine tubercle bacilli develop hypersensitiveness to an intracutaneous injection of tuberculin. This sensitiveness appears from the 2nd to the 6th week after infection and increases rapidly thereafter. Tests, as a rule, show fluctuation in the intensity of the sensitization. Sensitization is followed by an interval of several weeks preceding death during which the animals fail to react.

2. Rabbits infected with bovine tubercle bacilli form antibodies that fix complement in the presence of tubercle bacilli. The antibodies

appear after 2 weeks, increase during 6 to 10 weeks, and persist until the animals die.

3. In the later period of infection the skin fails to react to tuberculin at a time when the serum contains complement fixing antibodies.

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LIPIDS AND IMMUNOLOGICAL REACTIONS

III. LIPID CONTENT OF SPECIFIC PRECIPITATES FROM TYPE I ANTIPNEUMOCOCCUS SERA

BY FRANK L. HORSFALL, JR., M.D., AND KENNETH GOODNER, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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It has been reported (1, 2) that the presence of certain lipids is essential to the *in vitro* demonstration of type-specific agglutination and precipitation by antipneumococcus sera. It was suggested that the antibody of antipneumococcus serum might be a phosphatide-globulin complex, the nature of the lipid fraction being dependent upon the species derivation of the antibody. Because of the minute amounts of phosphatide necessary to restore the *in vitro* properties of antisera from which lipids had been extracted, it was to be expected that the amount of lipid actually associated with the antibody itself would be very small.

It is apparent that if lipid does form a portion of the antipneumococcus antibody, much the simplest means of defining both its amount and character would be by a study of the antibody in an isolated and pure condition. Since, however, technical difficulties which interfere with the isolation of pure antibodies have not been entirely surmounted, it was necessary to study the antibody in its native state. While the method adopted has the disadvantages inherent to the chemical study of a substance dispersed in a complex system, these disadvantages are to some extent offset by the fact that the antibody had not been altered by chemical manipulation. Perhaps the most convenient and satisfactory means of obtaining a separation of the antibody from the various constituents of antipneumococcus sera is by precipitation with the homologous capsular polysaccharide. Such precipitates, if carefully prepared and washed by the method of Heidelberg and Kendall (3), afford adequate and readily available material for the quantitative study of those lipids associated with type-specific

antipneumococcus reactions. It is obvious that in precipitates prepared from a solution as complex in composition as serum, one might find not only those very minute amounts of lipid assumed to be intimately associated with the antibody, but also any lipid which was adsorbed during the formation of the precipitate.

The literature on this subject is very meager. Douglas and Dudley (quoted by Hartley (4)) found that 25 per cent of a dried immune precipitate prepared from a mixture of horse serum and the corresponding rabbit antiserum was ether-soluble. Marrack and Smith reported (5) that diphtheria toxin-antitoxin floccules contained a small amount of chloroform-soluble material, but they were unable to find lipids in rabbit-anti-horse precipitates (6). Breinl and Haurowitz (7) found from 2 to 8 per cent lipid in hemoglobin-antihemoglobin precipitates, but were not able to detect cholesterol or phosphatides by the methods used. It is apparent that there is a diversity of opinion as to the presence or absence of lipid in specific precipitates.

It is the purpose of this paper to report a series of quantitative studies upon a large number of specific precipitates prepared under varying conditions from Type I antipneumococcus horse and rabbit serum.

Methods

One lot each of unconcentrated, monovalent horse and rabbit Type I antipneumococcus sera have been used as an antibody source. The sera were passed through Berkefeld V filters just prior to each experiment, and were then whirled for 30 minutes in the angle centrifuge at 0°C. in order to eliminate any particulate matter. The acetyl capsular polysaccharide of Type I Pneumococcus was used as the specific precipitating agent. This polysaccharide contained 4.4 per cent of acetyl and 5 per cent of nitrogen.

Experiments were carried out by two methods: (a) the addition of increasing amounts of polysaccharide to constant amounts of serum, and (b) the addition of constant amounts of polysaccharide to increasing amounts of serum. In the majority of experiments the total volume of each reacting mixture, *i.e.*, serum, saline, and polysaccharide, was 5.0 cc.

Precipitates were prepared and washed according to the method of Heidelberger and Kendall (3). The sera, accurately diluted with 0.9 per cent NaCl, were measured from a calibrated burette into chemically clean test tubes with an inside diameter of 1.2 cm. The serum-saline solutions were cooled to 0°C. in an ice bath, and cold polysaccharide solution in 0.9 per cent NaCl was then added.

The two solutions were immediately and thoroughly mixed by twirling. After standing at 4°C. for 24 hours, the precipitates were firmly packed by whirling in the angle centrifuge in the cold, and were washed twice with ice cold saline. Care was taken to break up the precipitates thoroughly during each washing in order to facilitate the removal of extraneous substances. In each instance precipitates were prepared in quadruplicate in order that one set of duplicates could be analyzed for total nitrogen and the other for both lipid carbon and lipid nitrogen.

For total nitrogen studies, duplicate precipitates were dissolved in small amounts of $N/10$ NaOH and the resulting solutions were then washed quantitatively into pyrex digestion tubes. The digestions and gasometric micro Kjeldahl analyses were carried out by the method of Van Slyke (8). Selenium catalyst was used in the digestion mixture as recommended by Kirk, Page, and Van Slyke (9). This method is accurate to 0.002 mg. of nitrogen. Duplicate analyses were made on each single precipitate by using aliquots of the neutralized digest. In the various tables below, the figures given for total nitrogen, unless otherwise indicated, are the mean values of four analyses (*viz.*, two aliquots on each of two duplicate precipitates). The mean variation between the values for determined nitrogen on aliquots from the same precipitates was 0.002 mg., while the mean variation of the determined nitrogen on duplicate precipitates was 0.005 mg. For the calculation of protein the corrected protein nitrogen was multiplied by the factor 6.25. The calculation of corrected protein nitrogen will be described below.

For the determination of total lipid carbon and lipid nitrogen, duplicate precipitates were thoroughly broken up in alcohol-ether 3:1 (both redistilled). The granular suspensions of the specific precipitates thus formed were transferred quantitatively to 100 cc. reflux flasks. This was made possible by means of ten separate washings with alcohol-ether. The remainder of the extraction method followed that of Kirk, Page, and Van Slyke (9). Because of the relatively small amounts of lipid in the precipitates, the secondary extraction of the dried alcohol-ether residue by petrol-ether was slightly altered, so that the final volume after filtration was only 50 cc. Two 10 cc. aliquots of the petrol-ether extract were transferred into combustion tubes for lipid carbon analyses, and a 20 cc. sample was pipetted into a pyrex digestion tube for lipid nitrogen analysis. For lipid carbon determinations the manometric micro method of Van Slyke, Page, and Kirk (10) was followed. This method is accurate to 0.003 mg. of carbon. The values for lipid carbon in the various tables below represent the mean quantity found in four analyses (*viz.*, two aliquots on each of two duplicate precipitates). The mean variation between the determined lipid carbon of aliquots from the same precipitate was 0.003 mg., and the mean variation of determined lipid carbon on duplicate precipitates was 0.004 mg. For the calculation of total lipid from lipid carbon the latter figure has been multiplied by the factor 1.3. This factor implies a mixture of lipids having an average carbon content of 77 per cent, and is an approximation for serum lipids.

Lipid nitrogen was determined by the method of Kirk, Page, and Van Slyke (9). In the various tables below lipid nitrogen figures represent the mean of separate

analyses upon duplicate precipitates. The mean variation between the determined lipid nitrogen of duplicate precipitates was 0.002 mg.

All determinations were carried out in the Van Slyke-Neill manometric apparatus.

Comparative Lipid Content of Specific Precipitates Prepared from Constant Amounts of Immune Serum and Varying Amounts of Capsular Polysaccharide

In order to determine the relation between the lipid and protein content of precipitates, analyses were carried out upon a large number

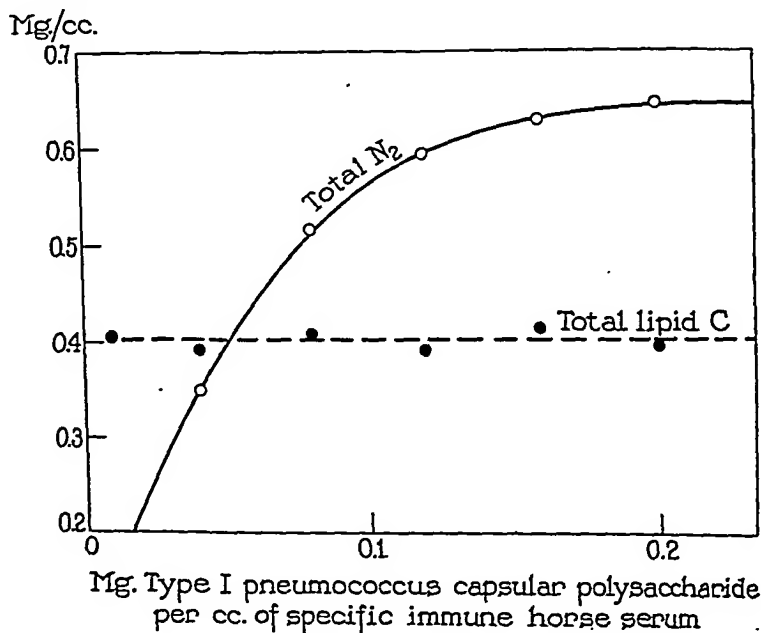
TABLE I
Total Nitrogen, Lipid Carbon, and Lipid Nitrogen of Specific Precipitates Formed from Constant Amounts of Immune Serum and Varying Amounts of Capsular Polysaccharide

Type I antipneumococcus serum		Type I capsular polysaccharide		Specific precipitate				
Species	Amount	Mg. per cc. of antiserum	N ₂ (calculated)	Total N ₂	Lipid N ₂	Protein N ₂ (calculated)	Lipid C	Lipid content of precipitate
	cc.		mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.	per cent
Horse	1.50	0.010	0.0005	0.157	0.021	0.136	0.405	38.2
"	1.50	0.040	0.002	0.350	0.015	0.333	0.392	19.7
"	1.50	0.080	0.004	0.518	0.007	0.507	0.411	14.4
"	1.50	0.120	0.006	0.594	0.018	0.572	0.390	12.4
"	1.50	0.160	0.008	0.628	0.013	0.611	0.417	12.4
"	1.50	0.200	0.010	0.645	0.017	0.624	0.398	11.7
Rabbit	1.50	0.017	0.0008	0.079				
"	1.50	0.067	0.003	0.281	0.012	0.266	0.385	23.1
"	1.50	0.133	0.006	0.500	0.008	0.486	0.392	14.4
"	1.50	0.200	0.010	0.636	0.012	0.618	0.380	11.3
"	1.50	0.267	0.013	0.673	0.011	0.656	0.388	10.9
"	1.50	0.333	0.016	0.656				

formed from constant amounts of horse and rabbit Type I antipneumococcus sera to which varying amounts of polysaccharide had been added. The results of experiments of this character are shown in Table I.

It will be noted that under the conditions of these experiments the lipid carbon content in each series of precipitates remained practically constant over the range studied, and bore no relationship to the total nitrogen. Furthermore, it will be

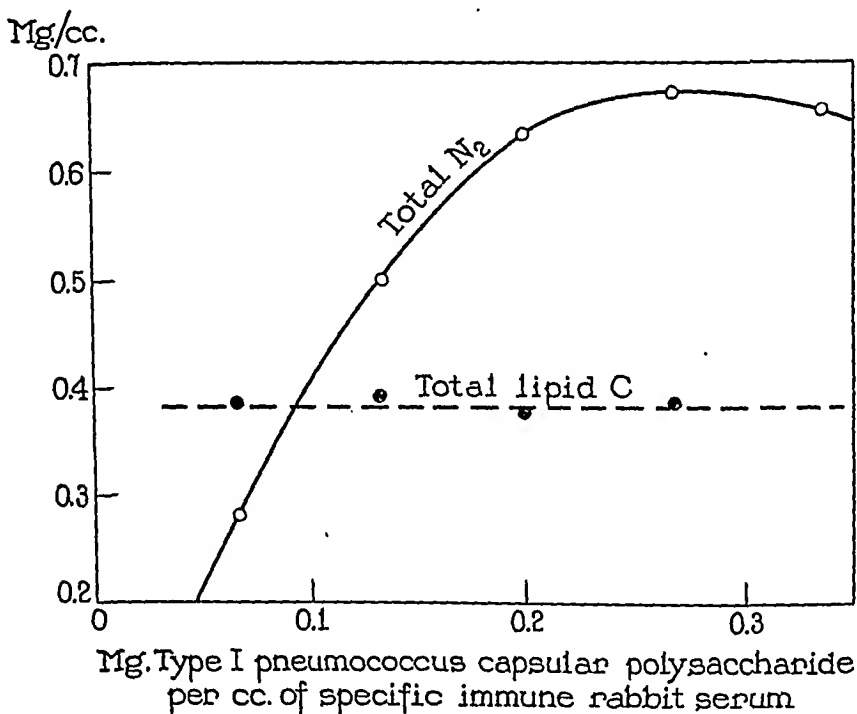
observed that the lipid nitrogen content of the precipitates remained almost constant and was not related to the total nitrogen content. As the amount of polysaccharide was increased the total nitrogen of the precipitates increased until a zone of maximum protein precipitation was reached. Upon the addition of still greater amounts of polysaccharide a gradual decrease in the total nitrogen occurred. These latter findings have been previously demonstrated by Heidelberger and Kendall (11) in a large number of careful experiments.



TEXT-FIG. 1. Total nitrogen and total lipid carbon of specific precipitates prepared from constant amounts of immune horse serum and varying amounts of capsular polysaccharide.

By plotting both the total nitrogen and the lipid carbon of these precipitates against the amount of polysaccharide per unit volume of serum, curves can be obtained which demonstrate graphically the constancy of the lipid content of precipitates prepared from constant amounts of antiserum. These curves, for both antipneumococcus horse and rabbit sera, are shown in Text-figs. 1 and 2, respectively. The absence of a direct relationship between the amounts of lipid carbon and the amounts of total nitrogen in these precipitates is clearly shown.

It must be pointed out that the total nitrogen of specific precipitates from Type I antipneumococcus horse or rabbit serum represents the sum of at least three separate nitrogen values. It is true that much the greater part of the total nitrogen is protein nitrogen. However, since the Type I polysaccharide contains 5 per cent of nitrogen, and a certain amount of lipid nitrogen has been shown to be present in the precipitates, these small quantities must be subtracted from the total nitrogen if an exact estimation of protein nitrogen is to be obtained.



TEXT-FIG. 2. Total nitrogen and total lipid carbon of specific precipitates prepared from constant amounts of immune rabbit serum and varying amounts of capsular polysaccharide.

It is possible in the region of antibody excess to calculate the polysaccharide nitrogen in the precipitates with considerable accuracy. In the region of antigen excess the amount of polysaccharide nitrogen can only be estimated, since the antigen content of the supernatants was not determined quantitatively. The figures for the calculated total polysaccharide nitrogen are shown in Table I. Corrected protein nitrogen was calculated by subtracting lipid nitrogen and polysaccharide nitrogen from the total nitrogen. The protein content of the precipitates was calculated by multiplying the corrected protein nitrogen by the factor

6.25. By the addition of the figures for protein and for lipid, that part of the total mass of the precipitate derived from the immune serum was obtained. From these figures the relative content of lipid was calculated.

From these results it will be noted that lipid may constitute a very considerable portion of the precipitate mass. In certain other instances, not indicated in the tables, lipid has been found to form as much as 51.2 per cent or as little as 3.8 per cent of the total precipitate mass. Heidelberger and Kendall (11) have reported a slight discrepancy between the observed values for total nitrogen of specific Type III precipitates prepared from unconcentrated sera and the calculated values as determined by their formulae. It may be pointed out that lipid nitrogen, although constituting a very small proportion of the total nitrogen, does introduce a slight correction factor with respect to protein nitrogen. This correction would tend to reduce the above noted discrepancy between observed and calculated values for total nitrogen, if one assumes that the precipitates studied contained amounts of lipid similar to those here reported.

*Comparative Lipid Content of Specific Precipitates Prepared from
Varying Amounts of Immune Serum and Constant
Amounts of Capsular Polysaccharide*

In order to extend and confirm the foregoing observations upon the lipid content of specific precipitates, additional experiments were carried out in which a large number of precipitates were formed by the addition of constant amounts of polysaccharide to increasing amounts of immune serum. The results of these experiments are shown in Table II.

It will be noted that the lipid carbon content of the precipitates expressed in terms of unit serum volume progressively and rapidly increased as the amount of antiserum was decreased. As in the previous experiments, there was no direct relation between the lipid carbon and the total nitrogen content of these precipitates. The lipid nitrogen content of the precipitates, calculated on the same basis, increased in a manner similar to the increase in lipid carbon, and was not related to the total nitrogen content of the precipitates. The quantity of total nitrogen in the precipitates in terms of unit serum volume increased to a maximum as the proportion of polysaccharide became greater, in a manner exactly comparable to that of the previous experiments.

Polysaccharide nitrogen, corrected protein nitrogen, and the relative lipid content of each precipitate have been calculated in the manner described above.

By plotting the total nitrogen and the lipid carbon values against the amounts of polysaccharide in milligrams per cubic centimeter of antiserum, curves are developed as shown in Text-figs. 3 and 4. These figures clearly demonstrate the absence of any direct relationship between the protein and lipid content of specific precipitates as prepared by this method. They also indicate that, in terms of unit serum volume, the lipid content of these precipitates is quite different from that of precipitates prepared by the previous method.

TABLE II

Total Nitrogen, Lipid Carbon, and Lipid Nitrogen of Specific Precipitates Formed from Varying Amounts of Immune Serum and Constant Amounts of Capsular Polysaccharide

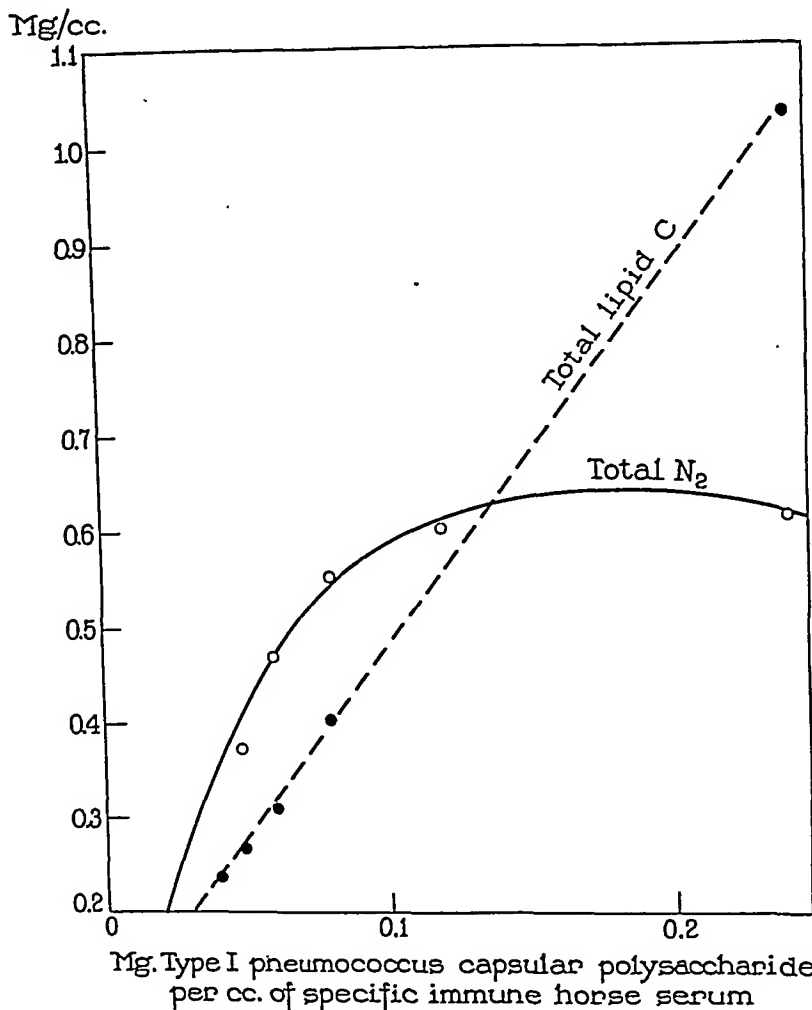
Type I antipneumococcus serum		Type I capsular polysaccharide		Specific precipitate				
Species	Amount	Mg. per cc. of antiserum	N ₂ (calculated)	Total N ₂	Lipid N ₂	Protein N ₂ (calculated)	Lipid C	Lipid content precipitate
	cc.		mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.	mg. per cc.	per cent
Horse	3.0	0.040	0.002	0.348	0.008	0.338	0.238	12.8
"	2.5	0.048	0.002	0.371	0.010	0.359	0.266	13.4
"	2.0	0.060	0.003	0.466	0.012	0.451	0.311	12.5
"	1.5	0.080	0.004	0.558	0.017	0.537	0.404	13.5
"	1.0	0.120	0.006	0.607	0.025	0.578	0.580	17.3
"	0.5	0.240	0.012	0.619	0.009	0.606	1.035	26.2
Rabbit	3.0	0.080	0.004	0.338*	0.005	0.329	0.213	11.9
"	2.0	0.120	0.006	0.460*	0.008	0.446	0.294	12.0
"	1.0	0.240	0.012	0.660*	0.015	0.639	0.518	14.4

* Interpolated from Text-fig. 2.

Factors Determining the Lipid Content of Immune Precipitates

From the foregoing results it is apparent that the amount of lipid in the immune precipitate bears no relationship to the amount of precipitated protein. This fact suggested the possibility that much of the lipid may be present in the precipitate as the result of adsorption. In order to explore this possibility, certain calculations have been applied to the observed data to determine if the amount of lipid in the precipitate bore any relationship to the concentration of lipid in the reacting mixture.

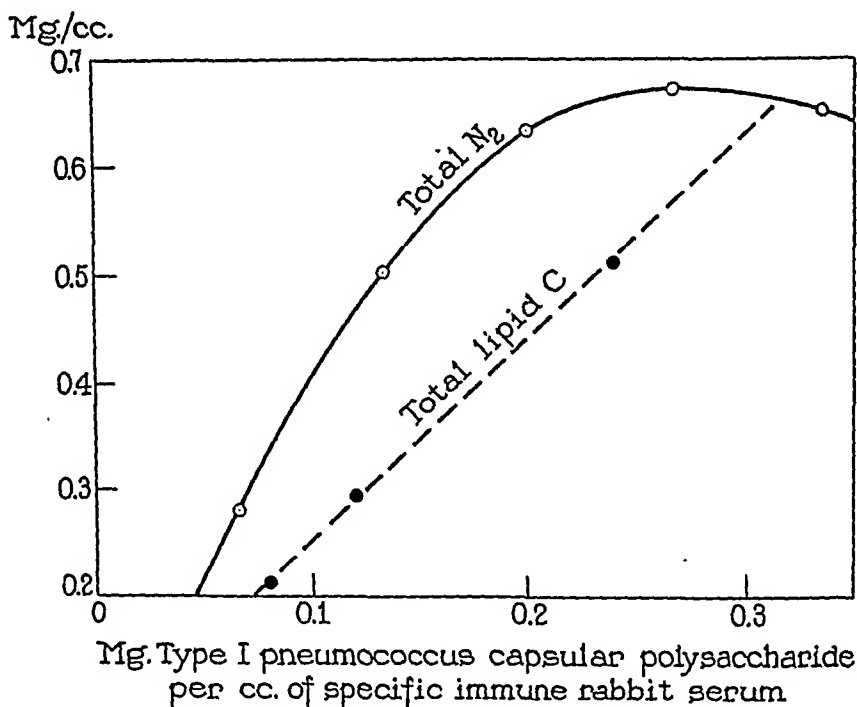
In Tables I and II, lipid carbon has been expressed in terms of unit serum volume. If, however, the total lipid carbon of each precipitate



TEXT FIG. 3. Total nitrogen and total lipid carbon of specific precipitates prepared from varying amounts of immune horse serum and constant amounts of capsular polysaccharide.

is expressed in its absolute value, irrespective of the amount of serum used, it becomes apparent that this value is definitely related to the amount of serum in those instances in which the total volume of the

reacting mixture has been kept constant. These relationships are shown in Table III. It will be noted that with a constant amount of serum the lipid carbon of the precipitates is constant, but that with progressively increasing serum volume the lipid carbon of the precipitates also progressively increases. This is true in precipitates formed both from rabbit and from horse immune serum.



TEXT-FIG. 4. Total nitrogen and total lipid carbon of specific precipitates prepared from varying amounts of immune rabbit serum and constant amounts of capsular polysaccharide.

In the majority of instances listed in Table III, the total volume of the reacting mixture was 5.0 cc., irrespective of the amount of antiserum or of polysaccharide used. In these experiments, then, it can be taken that the concentration of lipid in the reacting mixture is directly related to the volume of antiserum. The total lipid carbon content of the horse antiserum was 1.942 mg. per cc., and of the rabbit antiserum 1.737 mg. per cc. From these values and those given in Tables I and II, it is possible to derive the values given in Table III for the concentration of lipid carbon in the reacting mixture and the absolute lipid carbon content of the precipitates.

From a consideration of these data, it appears that the lipid carbon content of the specific precipitates is a function of the concentration of

TABLE III

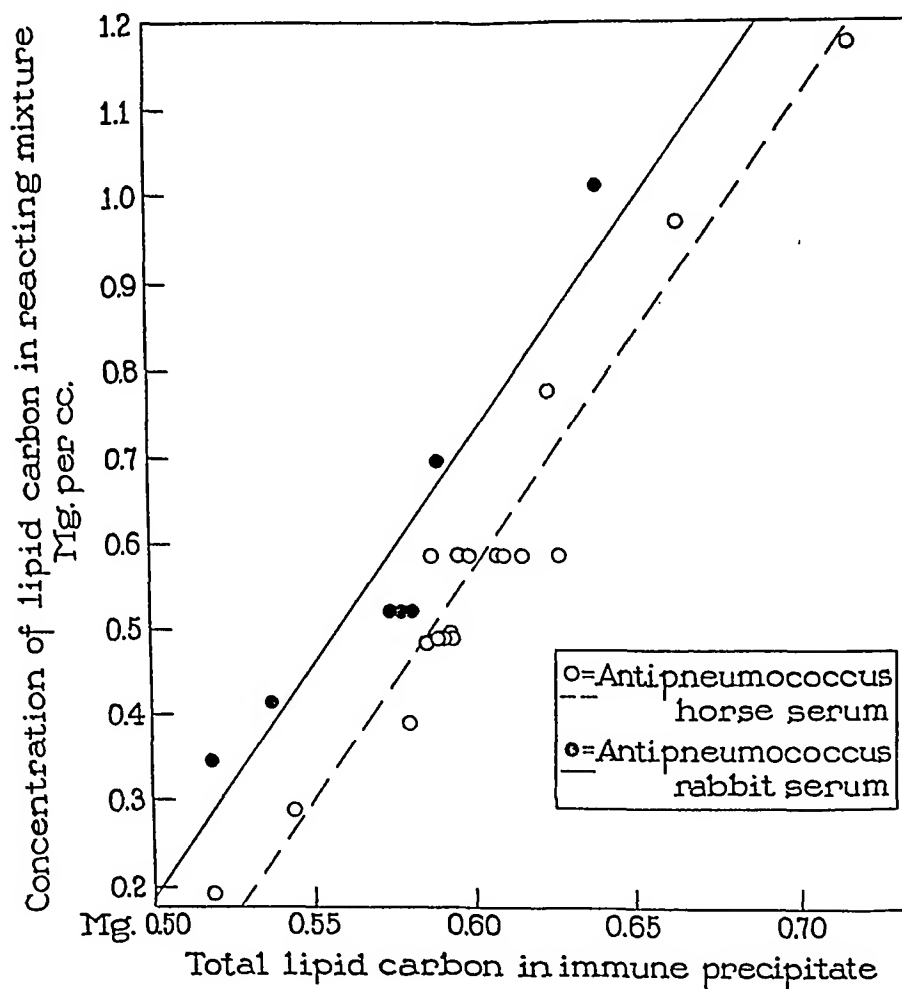
The Relation of the Lipid Content of Specific Precipitates to the Concentration of Lipids in the Reacting Mixtures

Type I antipneumococcus serum		Capsular polysaccharide	Total volume of reacting mixture	Total lipid C in reacting mixture	Concentration of lipid C in reacting mixture	Specific precipitate		Calculated lipid C of precipitate (formula 1)	Deviation of found from calculated
Species	Amount					Total N ₂ of precipitate	Total lipid C of precipitate		
	cc.	mg.	cc.	mg.	mg. per cc.	mg.	mg.	mg.	mg.
Horse	1.5	0.015	5.0	2.914	0.583	0.235	0.608	0.606	0.002
"	1.5	0.060	5.0	2.914	0.583	0.525	0.588	0.606	0.018
"	1.5	0.120	5.0	2.914	0.583	0.777	0.616	0.606	0.010
"	1.5	0.180	5.0	2.914	0.583	0.892	0.585	0.606	0.021
"	1.5	0.240	5.0	2.914	0.583	0.942	0.628	0.606	0.022
"	1.5	0.300	5.0	2.914	0.583	0.967	0.597	0.606	0.009
"	3.0	0.120	5.0	5.826	1.165	1.044	0.715	0.702	0.013
"	2.5	0.120	5.0	4.856	0.971	0.927	0.664	0.676	0.012
"	2.0	0.120	5.0	3.884	0.777	0.932	0.623	0.639	0.016
"	1.5	0.120	5.0	2.914	0.583	0.837	0.607	0.606	0.001
"	1.0	0.120	5.0	1.942	0.388	0.607	0.580	0.562	0.018
"	0.5	0.120	5.0	0.971	0.194	0.309	0.518	0.490	0.028
"	1.5	0.093	6.0	2.914	0.486		0.593	0.588	0.005
"	1.5	0.093	6.0	2.914	0.486		0.590	0.588	0.002
"	1.5	0.093	6.0	2.914	0.486		0.589	0.588	0.001
"	1.5	0.093	6.0	2.914	0.486		0.593	0.588	0.005
"	1.5	0.090	10.0	2.914	0.291		0.543	0.544	0.001
"	3.0	0.046	12.0	5.826	0.485	0.756	0.584	0.588	0.004
Rabbit	1.5	0.10	5.0	2.606	0.521	0.421	0.578	0.566	0.012
"	1.5	0.20	5.0	2.606	0.521	0.750	0.588	0.566	0.022
"	1.5	0.30	5.0	2.606	0.521	0.954	0.570	0.566	0.004
"	1.5	0.40	5.0	2.606	0.521	1.009	0.583	0.566	0.017
"	3.0	0.24	5.0	5.211	1.041	1.014*	0.639	0.643	0.004
"	2.0	0.24	5.0	3.474	0.695	0.920*	0.589	0.597	0.008
"	1.0	0.24	5.0	1.737	0.347	0.660*	0.518	0.522	0.004
"	3.0	0.375	12.0	5.211	0.434	1.551	0.535	0.545	0.010

* Interpolated from Text-fig. 2.

lipid in the reacting mixture. To test the validity of this relationship, other precipitates were prepared in the equivalence zone, using various

amounts of serum and total volumes of reacting mixtures which ranged from 5 to 12 cc. The values for lipid carbon found in these precipitates, as well as the total amount and concentration of lipid carbon in the reacting mixtures, are also given in Table III.



TEXT-FIG. 5. Relation of absolute lipid content of specific precipitates to initial lipid carbon concentration of reacting mixture.

The absolute lipid carbon content of the various precipitates from horse and rabbit antiserum, irrespective of the method of preparation or the total volume of the reacting mixture, have been plotted in Text-fig. 5 against the initial concentration of lipid carbon. Two straight

lines have been empirically inserted in Text-fig. 5 to indicate possible linear relationships between the lipid carbon content of precipitates and the concentration of lipid carbon in the reacting mixtures. Within the relatively narrow range covered by these experiments, these two lines appear to agree with the experimentally determined points fairly well. However, no extension of the lines shown is justified, for should they be extended it will be observed that they would intercept the zero concentration axis at 0.46 and 0.49 mg. respectively, an obviously impossible circumstance.

Since the lipid carbon in a specific precipitate is independent of the amount of the precipitate within the limits studied, but is a function of the initial concentration of lipid carbon in the reacting mixture, it seems reasonable to assume that much the greater part of the lipid present in a specific precipitate may be carried down as a result of adsorption.

It has been found that these data agree well with the Freundlich adsorption equation $\frac{y}{a} = kx^n$, in which $\frac{y}{a}$ = amount adsorbed per unit of surface, and x^n = a function of the concentration. Quite obviously, no calculation of the surface area of the precipitating particles is permissible, particularly since this surface progressively and rapidly decreases from the moment of the initial reaction between antigen and antibody to the time when the almost solid precipitate has settled out. Because of this difficulty it has been necessary to disregard the actual surface area of the forming precipitate, and to make the not inconsiderable assumption that it had in each instance a unit value. It has been found that the equation, under these circumstances, has the form:

$$y = kx^{0.2} \quad (1)$$

For the horse antiserum studied, k has been found to be 0.680, while for the rabbit antiserum $k = 0.645$. It is possible, by using this equation, to calculate the expected lipid carbon content of a single precipitate when the concentration of lipid carbon in the reacting mixture is known. These calculations have been carried out, and the results are shown in Table III. The deviation of the calculated from the observed values for the lipid carbon content of the precipitates has also been determined. The mean deviation of these values for precipitates from either horse or rabbit antiserum is ± 0.010 mg. In the case of immune horse serum, this amounts to ± 1.6 per cent, and to ± 1.7 per cent in the case of rabbit antiserum.

Lipid Content of Successive Precipitates from the Same Portion of Antiserum

In the preceding sections it has been demonstrated that the lipid content of any single specific precipitate from antipneumococcus horse or rabbit serum is a function of the concentration of lipid in the reacting mixture before precipitation occurs. In these instances only a single precipitate was produced with each portion of antiserum. It is possible, however, to produce a number of precipitates from any given amount of antiserum by the consecutive addition of small amounts of

TABLE IV

Lipid Content of Specific Precipitates Prepared Successively from the Same Portion of Antiserum

Experiment	Precipitate	Type I antipneumococcus serum		Capsular polysaccharide	Total volume of reacting mixture	Total lipid C in reacting mixture	Concentration of lipid C in reacting mixture	Specific precipitate		Calculated lipid C of precipitate (formula 1)	Deviation of found from calculated
		Species	Amount					Total N ₂ of precipitate	Total lipid C of precipitate		
			cc.	mg.	cc.	mg.	mg. per cc.	mg.	mg.	mg.	mg.
A	1	Horse	2.5	0.120	5.0	4.856	0.971	0.927	0.664	0.676	0.012
	2	"	1.5	0.015	4.66	2.517	0.540	0.134	0.431	0.601	0.170
	3	"	0.965	0.008	5.0	1.343	0.269	0.043	0.035	0.523	0.488
B	1	"	3.0	0.120	5.0	5.826	1.165	1.044	0.715	0.702	0.013
	2	"	2.0	0.020	5.0	3.063	0.612	0.196	0.475	0.616	0.141
	3	"	1.0	0.008	5.0	1.551	0.310	0.046	0.434	0.538	0.104

polysaccharide. It was important, therefore, to determine the lipid content of consecutively formed precipitates. The results of two experiments, in each of which three separate precipitates were formed from the same portion of antiserum, are presented in Table IV.

It will be noted that in each experiment the lipid carbon content of the second and third consecutively formed precipitates was considerably less than that of the first precipitate. This reduction in lipid in the precipitates was considerably greater than the diminution in the concentration of lipid in the reacting mixtures. The extent of this reduction becomes more apparent when the observed lipid carbon values for the second and third precipitates are compared with values calculated by formula 1. Although, as shown in Table III, there is a close cor-

relation between the observed and calculated lipid carbon values for an initial precipitate, this relationship is considerably altered in the second and third consecutively formed precipitates. In the latter there was at least 0.10 mg. less lipid carbon than would be anticipated by application of the formula used for initial precipitates. This result was to be expected, since it is likely that those lipids most readily adsorbed would be carried down by the first precipitate, thus leaving somewhat less easily adsorbed lipids in the supernatant. Under these circumstances the second and the third precipitates would be formed in the presence of lipid which was less and less readily adsorbable, and consequently should contain amounts of lipid considerably smaller than those found in initial precipitates. These considerations are borne out by the experimental observations.

It must be pointed out that in these experiments the nature of the various lipids present in the reacting mixtures, as well as in the precipitates, has been disregarded. It is obvious that a number of different lipids (*i.e.*, cholesterol, cholesterol esters, phosphatides, neutral fats, and fatty acids) are present in the immune sera and the distinctive properties of these various substances suggest that some may participate in adsorptive processes to a greater extent than others.

DISCUSSION

The initial purpose of this study was to attempt the quantitative estimation of those lipids which previous work (1) had suggested might be intimately associated with the antibody globulin of Type I anti-pneumococcus sera. Inasmuch as complete isolation of the antibody has not yet been achieved, it was impossible to approach the problem by direct analysis. It was determined, therefore, to study the anti-pneumococcus antibody as it occurs in specific precipitates resulting from the interaction of antisera and the homologous pneumococcus capsular polysaccharide.

The specific precipitates were carefully prepared and washed by the method of Heidelberger and Kendall (3) and it was found that they contained a relatively large amount of lipid. In some, as much as 51 per cent, in others as little as 4 per cent by weight of the total precipitate was lipid. These quantities were very much greater than the amounts which had been anticipated, particularly when it is recalled that only very small amounts of phosphatide were required to restore certain *in vitro* properties to antisera extracted by lipid solvents (1).

Not only was the total lipid content of the precipitates greater than might be expected, but there was no stoichiometric relation between the lipid and protein contents of the precipitates. Were all the lipid

of specific precipitates present only by virtue of an assumed intimate association with antibody globulin, a constant ratio should exist between the amounts of protein and lipid occurring in the precipitates. Under these circumstances, too, quantitative curves for the protein and lipid content of variously prepared precipitates should have the same form. That they do not is manifest in each of Text-figs. 1 to 4. Within the ranges studied, the total lipid content of specific Type I precipitates was entirely independent of the protein.

The total lipid content of specific precipitates was found to be a function of the initial concentration of lipid in the reacting mixture prior to the formation of the precipitate. Since certain adsorption phenomena manifest a linear relationship between the quantity of a substance adsorbed and some exponential function of its concentration in the system, the analytical results were compared with the various calculated results. The Freundlich adsorption equation: $y = kx^n$ was applied, and it was found that if the exponent n were taken to be 0.2, the observed values for y (the lipid carbon of the precipitates) had a mean deviation from the calculated values of but 0.01 mg. Although this correlation between calculated and observed values for the lipid carbon content of specific precipitates may be considered as significant, it is thought that the experimental findings scarcely warrant a definite statement that the Freundlich equation describes the relationship over the entire course of the reaction.

The fact that considerable amounts of lipid have been regularly found in Type I specific precipitates and that the quantities appear to bear a linear relationship to an exponential function of the concentration of lipid initially in the systems, neither lends weight to, nor does it invalidate the conception that lipid may be intimately associated with the antibody globulin. If, for example, one assumes that the ratio of protein to lipid in the antibody is roughly 100:1, a not unreasonable assumption in the light of the comparative molecular weights of globulin and phosphatide, and also because of the small quantity of phosphatide necessary to restore *in vitro* properties to extracted antiserum, it is possible to approximate the amount of supposed antibody-lipid in each precipitate. When this is done it is found that even in the case of the heaviest precipitate studied (*i.e.*, 0.656 mg. protein nitrogen per cc.) the associated antibody-lipid

carbon would have a value of but 0.031 mg. per cc. In the same precipitate the total lipid carbon content was found to be 0.388 mg. per cc. In the case of smaller precipitates this associated antibody-lipid carbon would be proportionately less. Under these circumstances, and if the necessary assumptions are in any way an indication of what actually holds, it is easily conceivable that these small though significant quantities of lipid would be masked by the relatively large amounts of additional lipid found, and therefore do not seriously affect the apparent correlation between observed lipid carbon content and that calculated on the basis of adsorption.

SUMMARY

1. Specific precipitates resulting from the interaction of the homologous capsular polysaccharide and Type I antipneumococcus horse and rabbit sera have been analyzed by gasometric micro methods for total nitrogen, lipid nitrogen, and lipid carbon.

2. Lipid may, under certain conditions, form as much as 51 per cent or as little as 4 per cent by weight of specific precipitates.

3. The total lipid content of specific precipitates, within the range studied, is entirely independent of the protein content.

4. Lipid nitrogen forms a very small but detectable portion of the total nitrogen of precipitates.

5. The absolute lipid content of precipitates is a function of the concentration of lipid in the reacting mixture prior to precipitation, and seems to be governed by the laws of adsorption phenomena.

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SPOTTED FEVER

II. AN EXPERIMENTAL STUDY OF FIÈVRE BOUTONNEUSE

By GEORGE M. HASS,* M.D., AND HENRY PINKERTON, M.D.

(From the Department of Pathology, The Harvard Medical School, Boston)

PLATES 35 TO 37

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There are three principal causes for the inconsistencies which exist among the classifications of the human rickettsial diseases and their etiologic agents. First, many investigators have drawn unwarranted conclusions from data obtained by one or two methods of study. Second, undue emphasis has been placed either upon minor differences among various strains of rickettsiae or slight variations in the diseases which they produce in man and experimental animals. Third, it has been difficult to construct useful classifications in the absence of rigorous standards for judging the significance of certain types of data.

In order to establish uniform methods of experimental procedure and analysis, one of us described several methods for the study of pathogenic rickettsiae (1). The data obtained by application of these methods were evaluated from the point of view of classification. This necessitated the proposal of certain criteria, so designed as to facilitate an analysis of the data. These differential criteria were selected on the basis of experience with several strains of pathogenic rickettsiae studied in this laboratory: European typhus, Breinl strain; Mexican typhus, Mooser strain; North Carolina typhus, Maxcy strain; Rocky Mountain spotted fever, Parker strain; Eastern spotted fever, Rumreich, Dyer, and Badger strain; Minnesota spotted fever, Reimann strain (2-7).

It was stated that a careful analysis of the available data permits the recognition of two genera of rickettsiae pathogenic for man. Furthermore, it was shown that only one species has been discovered in each genus, and that the differences between various strains in each

* Society of Fellows, Harvard University.

species must be regarded as of subspecific value. Consequently, the conclusion was reached that the name, *Rickettsia prowazeki*, originally given to the etiological agent of human typhus by da Rocha-Lima must be applied to the rickettsiae of all known diseases of the typhus fever group and the name *Dermacentroxenus rickettsi*, originally applied by Wolbach to the etiological agent of Rocky Mountain spotted fever must be applied to the rickettsiae which cause the diseases of the spotted fever group. The names typhus fever and spotted fever were selected to conform with those of the prototypal diseases, since distinguishing names are necessary for the designation of two such widely separated groups of diseases. There is some evidence that the mite-borne rickettsial diseases may form a third group and that the corresponding rickettsiae may belong to a third genus or species. However, at the present time the available experimental data are not adequate for the support of such distinctions.

We have recently completed a number of experiments with the rickettsiae proved by Caminopetros (8) to be the etiological agent of fièvre boutonneuse. In the present report, the data obtained from our studies have been combined with pertinent information available in the literature. The following aspects of fièvre boutonneuse and its etiological agent will be considered and the data will be subjected to analysis according to the standards previously referred to. 1. Clinical study of the disease in man. 2. Weil-Felix reaction. 3. Clinical study of the disease in guinea pigs. 4. Study of smears of scrotal secretion. 5. Histopathology in man and animals. 6. Location and morphology of microorganisms in paraffin sections of tissues of man and animal host. 7. Study of crossed immunity. 8. Study of the microorganisms in the arthropod vector. 9. Study of the microorganism in tissue culture.

Source of the Strains.—Through the courtesy of Dr. Caminopetros of the Pasteur Institute of Athens, three strains of the causative agent of fièvre boutonneuse have been available for our experiments. They have been isolated by the injection of the viscera of the common dog tick (*Rhipicephalus sanguineus*) intraperitoneally into guinea pigs. The ticks had been collected from dogs inhabiting regions of Greece and Morocco where fièvre boutonneuse is endemic. One strain has been isolated in this laboratory. Two strains kindly have been sent

to us by Dr. Parker. All strains have been maintained serially in guinea pigs by intraperitoneal injection of scrotal sac exudate. Although there has been a trend toward diminished virulence after the passage of one of these strains through a series of animals, we have noticed no fundamental differences among the three strains. Within the limits of the experimental data they must be considered as identical.

1. Clinical Study of the Disease in Man

Complete clinical descriptions have been made by Olmer (9) and Combiesco (10). A brief survey has been necessary for our purposes.

Conor and Bruch in 1910 (11) described an endemic disease of Tunis. They named it *fièvre boutonneuse*. Since the original account, numerous instances of diseases with a similar or identical clinical picture have been reported in many regions of the Mediterranean littoral. New names for the diseases have frequently accompanied these reports. Among them have been the following: *fièvre escharonodulaire*, *exanthème typhoïde estival*, *dothiendermie aiguë*, *exanthème infectieux épidémique*, *exanthematous fever*, *eruptive fever*, *Marseilles fever*, *fever of Conor and Bruch*, *typhus-like fever*, and *typhus fever*.

The malady may be defined as a tick-borne, rickettsial disease which usually has been characterized by a primary lesion at the site of the tick bite, an acute onset, mild febrile reaction, generalized exanthema, brief clinical course, and low mortality rate.

The majority of the cases have occurred in adults during the warm summer months. Rare instances have been reported in the early spring and late autumn.

A primary lesion, the *tache noire*, has been found in about one-half of the patients, usually at the site of a tick bite. The typical lesion has been an indurated, hyperemic, painless, and occasionally ulcerous papule, with regional lymphangitis. In a few instances the primary sore has been in the conjunctiva (12). It was assumed that the local infection in these patients was probably produced by introduction of the infectious agent into the eye by rubbing the eye with fingers contaminated with infectious viscera of crushed ticks. The primary lesion has been produced experimentally in man by subcutaneous or intracutaneous injection of blood obtained from human beings with the disease and by a similar injection of the viscera of infectious ticks (10).

The duration of the disease has usually been 12 to 14 days. During the first 2 to 3 days there have been nonspecific, mild, prodromal symptoms accompanied by an elevation of temperature to approximately 40°C. Within 3 or 4 days after the onset of fever, a maculopapular rash has appeared, first on the trunk and then, after spreading rapidly to the extremities, has involved the soles of the feet and the palms of the hands. The cutaneous lesions, pale red in color in the early stages, later have often become hemorrhagic and ulcerous. The fever has

subsided by lysis during the evolution of the rash. Except for a prolonged convalescence, complications have been few and unimportant.

The course of the disease in children has been brief and the symptoms mild. The rare fatalities have occurred in adults whose previous physical state was such that any mild infection would have been serious.

2. *Weil-Felix Reaction*

There have been numerous reports concerned with the results of the Weil-Felix reaction, conducted with human serum and several strains of *B. proteus*. The results have not been uniform and certain inconsistencies among the data of several investigators have caused some confusion. The discovery that the reaction varies among different patients as well as in the same patient during the course of the disease has aided in explaining some of the inconsistencies. We are inclined to accept the conclusions reached by Felix after he had corroborated Durand's careful studies of the Weil-Felix reaction in *fièvre boutonneuse* (13). The serum from patients with *fièvre boutonneuse* usually agglutinated *B. proteus* O X 19 in low titre. A lower titre and often no agglutination were obtained when *B. proteus* O X 2 and *B. proteus* O X K were used. Felix has interpreted the results as indicative of "group" agglutination, similar to that obtained in the study of Rocky Mountain spotted fever and tick bite fever. The "main" antigen has not been discovered.

Serological studies conducted by Caminopetros and Contos (14) have been concerned with the action of immune serum on the viable rickettsiac. They have found that the serum obtained from monkeys after the animals had been inoculated with nonviable rickettsiae of *fièvre boutonneuse* and also the serum of patients who were convalescing from *fièvre boutonneuse* neutralize the action of the viable rickettsiae of *fièvre boutonneuse*, while the serum of patients who had recovered from typhus fever does not neutralize the action of these microorganisms.

3. *Clinical Study of the Disease in Guinea Pigs*

The clinical manifestations which have followed the intraperitoneal injection of scrotal sac exudate or the viscera of ticks into guinea pigs have been fairly uniform. Within 2 to 5 days after injection, the temperature usually has reached 104–106°F. and scrotal edema and

hyperemia have developed. During this stage the presence of adhesions between the visceral and parietal layers of the tunica vaginalis may be detected by the mechanical resistance to the manual reduction of the testes into the peritoneal cavity. These adhesions, at first quite easily ruptured, later have become sufficiently organized to prevent an easy reduction of the testes into the peritoneal cavity. Within 3 or 4 days after the onset of edema and hyperemia of the scrotal sac, these manifestations have reached a maximum severity and subsided. The temperature has fallen by lysis and within 10 to 14 days after intraperitoneal injection has reached normal values. The animals have not been prostrated by the infection and the mortality rate has been negligible.

The principal variations from the typical findings have been in the length of the incubation period and the intensity of the scrotal inflammation. Rarely, the incubation period has been prolonged to 7 to 10 days. The severity of the scrotal reaction as produced by two strains has been fairly uniform. The behavior of the third strain has been different in two ways. First, the injection of the viscera of ticks has been followed by the rapid onset of high fever, symptoms of extreme prostration, and death within 2 days. Each animal had an acute generalized peritonitis. The exudate contained, in addition to numerous rickettsiae, a large bacillus. It has been shown that a pure culture of this bacillus causes a sudden fatal peritonitis, not only in normal guinea pigs but also in those which have recovered from *fièvre boutonneuse*. It has only been through a graded dilution of the emulsions of the viscera of ticks and through reinjection of small amounts of the exudate that the strain of rickettsiae was separated from this unusual contaminant. Secondly, the scrotal reaction produced by this strain of rickettsiae gradually has diminished in intensity after passage through a series of animals. This phenomenon apparently has been encountered by Caminopetros (14). He has stated that infectious cerebral tissue, if injected intraperitoneally, restores the original intensity of the reaction.

There has been a difference, clinically, between those animals injected intraperitoneally and those which have received infectious exudate subcutaneously. Although only a few animals have been injected subcutaneously, none has developed either a high fever or a

scrotal reaction. This type of response has been comparable to that of one animal upon which infectious ticks had fed. This animal had a mild fever and no scrotal reaction. Subsequently, it was found to be immune to an intraperitoneal injection of scrotal sac exudate.

4. *Study of Smears of Scrotal Sac Exudate*

The methods for microscopic study of scrotal sac exudate have been described in a previous report (3).

All stages of the inflammatory reaction have been examined. The most satisfactory preparations have been obtained on the 1st or 2nd day after the formation of adhesions between the two layers of the tunica vaginalis.

The direct smears, stained with Giemsa's solution, have had a variable ratio of polymorphonuclear leucocytes, macrophages, and serosal cells. Polymorphonuclear leucocytes have been numerous during the acute stage of exudation. Macrophages have been predominant in the period of organization and early repair.

Rickettsiae have been found in all preparations. Although they usually have been most numerous in the acute voluminous exudate, there has been no accurate correlation between the number of rickettsiae and the gross appearance of the exudate. Not infrequently a copious exudate obtained from an animal which had a severe scrotal reaction contained few rickettsiae. It has been common to find numerous microorganisms in a scanty exudate obtained from an animal which had a mild scrotal reaction.

The rickettsiae have been found in intracellular and extracellular locations. The majority of the microorganisms have been embedded in the cytoplasm of macrophages and serosal cells. In a few smears the nuclei of several cells have contained clusters of rickettsiae. Rarely, microorganisms have been found in the cytoplasm of polymorphonuclear leucocytes. The scattered extracellular rickettsiae seem to have gained this position principally as the consequence of the crushing of cells during the preparation of smears.

The microorganisms have never occurred in large numbers in the cytoplasm. In the most heavily infected cells, no more than 25 to 30 pairs have been present. As a rule, they have been scattered at

random, but an occasional compact cluster has been found, more frequently near the periphery of the cell than elsewhere.

The rickettsiae have had a distinctive morphology. The typical form has been lanceolate and the microorganisms have been associated characteristically in pairs. Spherical diplococcoid or linear diplobacillary forms have represented the usual extremes of morphological range. Isolated single microorganisms and an occasional short chain of 2 to 3 pairs have been found.

There has been a great variation in size. The large forms have been distinguishable under the magnification of a high dry objective. The smallest forms have been so minute that resolution has been difficult to achieve, even with the aid of a 1.3 mm. oil immersion lens and a perfect preparation.

The staining reaction with Giemsa's solution usually has been rather intense. The rickettsiae have been blue and sharply defined, with a narrow clear halo around them.

The intranuclear rickettsiae usually have occurred in one or more small clusters surrounded by a halo and imbedded in a pale blue, homogeneous nucleoplasm. Occasional nuclei have been filled with a compact mass of microorganisms. The average dimensions of intranuclear forms have been less than those of the intracytoplasmic microorganisms, although the extreme ranges have been equivalent.

5. *Histopathology in Man and Animals*

A few incomplete studies of the histopathology of the disease have been reported. Olmer (9) has examined the cutaneous lesions of Marseilles fever, a disease which is identical with *fièvre boutonneuse*. He described small accumulations of lymphocytes, monocytes, and polymorphonuclear leucocytes around vessels of precapillary and capillary dimensions. Combiesco (10) has found a similar reaction in the subcutaneous tissues. No rickettsiae have been demonstrated in the sections. Troisier and Cattani (15) have described perivascular cellular aggregates in a monkey with *fièvre boutonneuse*.

Inasmuch as no human tissues have been available for study in this laboratory, our histopathological data have been obtained from an examination of guinea pigs. The tissues have been treated by the Regaud-Giemsa technique, which has been previously described (3).

The brain, scrotal sac, epididymis, testis, and sites of the tick bite have been studied with special care. There have been no cerebral vascular lesions. The scrotal sac and its contents have shown an acute inflammation of the tunica, an exudate over the surface of the tunica, a thromboangeitis, and focal necroses. The continuity of the serosal cells frequently has been interrupted by small areas of acute necrosis. From these small abscesses a rather diffuse cellular infiltration has extended for variable distances beneath the partially intact but swollen serosal cells. The musculature and connective tissue of the scrotum have been involved but the testicular substance, except in perivascular regions, usually has been unaffected except for the cessation of spermatogenesis which always accompanies the inflammation with its attendant local elevation in temperature. The reaction has been accompanied by an exudate, distributed rather unevenly over the surface of the tunica. The exudate has been composed of a variable ratio of polymorphonuclear leucocytes, macrophages, and serosal cells. The acute thromboangeitis has been restricted to the small arteries and venules, especially those of the scrotal sac and the polar fat of the testis. The more severe lesions of vessels have been characterized by an acute segmental necrosis of the wall of the vessel and a fibrinous thrombus in the lumen. The necrosis usually has extended from the intima through the media into the adventitia. The structure of the intima and media has been replaced by cellular debris and a large number of polymorphonuclear leucocytes and macrophages. The cellular infiltration has been distributed throughout the periadventitial tissues and often has had an eccentric focal zone of concentration.

The microscopic sections of the cutaneous papules which developed at the sites of several tick bites have shown epithelial destruction, inflammation of the corium, extravasation of blood, thromboangeitis and rickettsiae. The epithelium over a small area has been replaced by a localized superficial acute inflammatory exudate in which bacteria of various types have been found. Throughout the corium and subcutaneous fat there has been a moderate number of macrophages, polymorphonuclear leucocytes, and extravasated red blood cells. The inflammatory cells have been most numerous around small blood vessels, several of which have shown partial necrosis of their walls and

thrombi in their lumina. The thrombi have been especially common in greatly dilated veins.

6. *Location and Morphology of the Microorganisms in Paraffin Sections of Tissues of Mammalian Host*

In several instances, the study of the microorganisms in paraffin sections has been more satisfactory than in smears. The general distribution of the organisms in the exudate has been similar to that in smears. Several intranuclear clusters have been found. Only a few rickettsiae have been present in the intact serosal cells. They have been demonstrated with some difficulty in the focal necroses of the tunica and in the endothelial and smooth muscle cells of blood vessels. The blood vessels in the region of the tick bite have contained more rickettsiae in their walls and intimal endothelial cells than those in the scrotum or polar fat of the testis. The morphology of the rickettsiae, whether intracytoplasmic or intranuclear, has been identical with that described in smears.

7. *Study of Crossed Immunity*

Several experiments concerned with the question of crossed immunity among fièvre boutonneuse and other rickettsial diseases in guinea pigs have been reported. Caminopetros and Contos (14) have found no crossed immunity between fièvre boutonneuse and typhus fever. Two strains of *R. prowazeki* representing European typhus and Brill's disease were used in their experiments. Brumpt (16) has concluded that fièvre boutonneuse does not protect against Rocky Mountain spotted fever. Badger (17) has obtained evidence of reciprocal crossed immunity between fièvre boutonneuse and Rocky Mountain spotted fever and has concluded that they are immunologically identical. Davis and Parker (18) have reported a positive crossed immunity among São Paulo typhus, Rocky Mountain spotted fever, and fièvre boutonneuse. Parker¹ has made the interesting observation that his Rocky Mountain spotted fever vaccine, prepared from infected ticks, does not protect guinea pigs against fièvre boutonneuse.

In order to evaluate the validity of a single contradiction to the generally uniform results, we have conducted a test for crossed immunity between fièvre boutonneuse and Rocky Mountain spotted fever. A strain of Rocky Mountain spotted fever which invariably has been fatal to normal guinea pigs was injected into normal guinea pigs. As soon as the animals had become moribund, blood was aspirated from the heart. Several cubic centimeters of this blood were then injected intraperitoneally into each of six guinea pigs which had recovered from

¹ Parker, R.R., personal communication.

fièvre boutonneuse several weeks previously. The animals did not react to the injection.

Our experiments with Parker's vaccine have confirmed his results. Each of twelve guinea pigs was injected with 2 cc. of Rocky Mountain spotted fever vaccine. Six of the vaccinated animals and six control animals were given intraperitoneal injections of 2 cc. of blood from animals moribund with Rocky Mountain spotted fever. The six vaccinated animals exhibited no significant evidence of disease. The control animals died of typical Rocky Mountain spotted fever.

The remaining six vaccinated animals were given, intraperitoneally, graded amounts of scrotal sac exudate and splenic tissue from animals ill with fièvre boutonneuse. Six normal unvaccinated controls were injected with the same graded quantities of infectious material. Five animals in each group developed fièvre boutonneuse. One vaccinated animal and the corresponding control, each of which had received the smallest amount of infectious tissue, did not have either a scrotal reaction or an elevation of temperature.

8. Study of the Microorganism in the Arthropod Vector

A series of observations and experiments by Caminopetros and others have proved beyond reasonable doubt that the common dog tick (*Rhipicephalus sanguineus*) is a vector of the causative agent of fièvre boutonneuse and that the dog is a natural reservoir. The clinical observations have associated the disease with the bite of this tick. It has been shown, in endemic areas, that the viscera of this variety of tick are infectious for several species of animals and that when injected into man, are productive, not only of the primary lesion, but also the typical clinical disease.

These facts have led us to undertake a study of 50 ticks (*Rhipicephalus sanguineus*) which had been collected from dogs owned by a man who had recently acquired fièvre boutonneuse. The methods which have been applied to this study are essentially the same as those developed by Wolbach (6).

The ticks were divided arbitrarily into two lots. One lot was used to establish the strain in guinea pigs and to determine by smears the distribution and morphology of the rickettsiae in the vector. For these purposes, each tick was dissected and smears made of portions of several organs. The remnants of the viscera were then crushed in a mixture of serum and Tyrode's solution. Graded dilutions of the pooled viscera were injected intraperitoneally into twelve guinea pigs. The second lot, composed of twenty-five ticks, was used for histologic study. This lot was divided into two groups. One group (eleven ticks) was dissected without having fed since removal from dogs in Greece, several weeks

previously. The second group (fourteen ticks) was allowed to feed on normal guinea pigs for 7 days. After incubation at room temperature for 10 to 14 days, they were dissected. During dissection, smears were made of the hypoderm, salivary glands, gut, and sexual organs. The viscera of each tick were subsequently fixed in Regaud's solution, embedded in paraffin, sectioned serially, and stained by the Giemsa method.

The smears of the organs of the ticks used for establishing the strain, and those of the ticks which also had not refed but which were studied in serial sections have been essentially identical. A comparison of the smears of the ticks not refed with those of the ticks refed in this laboratory has given evidence that multiplication of the rickettsiae has followed feeding and incubation. Inasmuch as the smears have given only a rough approximation to the more precise findings in serial sections, it is only necessary to present the data obtained from serial sections.

All of the ticks of the group which had not refed have contained rickettsiae. The serial sections have shown a great variation in the number of microorganisms, not only among the individual ticks but also in the various organs and parts of the organs in the same tick. In two ticks, almost all organs contained numerous rickettsiae, but usually the rickettsiae were fairly well localized and sparsely distributed. The cells of the gut, hypoderm, and ovaries have been the common sites of predilection. Localization in the cytoplasm has been constant but no intranuclear rickettsiae have been found in this series. Diplococcoid and diplobacillary forms frequently have been predominant. Many other structures, too numerous to mention, have exhibited such a range in morphology and staining reactions that they have not been considered as related to the rickettsiae under consideration, although it must be borne in mind that the morphological range of *Dermacentroxenus rickettsi* in the tick has not been completely determined.

A study of fourteen ticks which had refed prior to dissection has added several interesting facts. The guinea pig upon which they had fed acquired an immunity to fièvre boutonneuse. Three cutaneous papules at the site of tick bites showed the characteristic thrombo-angitis with rickettsiae in the walls and endothelial lining cells of blood vessels. The serial sections of these ticks in general have shown

rickettsiae in larger numbers and more diffusely distributed than those of the control series which had not refed. Furthermore, intranuclear rickettsiae have been found in four ticks. The cytoplasm of the cells of the gut, brain, hypoderm, ovaries, smooth muscle, and striated muscle frequently has contained microorganisms in large numbers. Usually they have been either isolated or in small clusters. Compact masses entirely replacing the cytoplasm have not been found. Small diplococcoid and diplobacillary forms have been especially numerous in those ticks in which intranuclear rickettsiae were demonstrable.

The intranuclear rickettsiae have been in several cells of the rectal pouch, the female sexual organs, striated muscle, Malpighian tubules, and hypoderm. These microorganisms have usually occurred in clusters in the center of the nucleus or uniformly distributed throughout the nucleus. In the latter instance, great distension of the nuclear membrane has been a common finding. Chromatin and nucleoli usually have either been wholly indistinguishable or persistent only at the nuclear membrane. When in compact masses the microorganisms usually have been smaller and more coccoid than when in small clusters. However, in both instances lanceolate diplococci and diplobacilli of relatively great size have been found.

9. Study of the Microorganisms in Tissue Culture

The plasma clot tissue culture method (4, 5) and the Maitland technique (19) as applied to the investigation of rickettsiae have previously been described. In this present study, scrotal sac exudate and infectious splenic tissue either singly or in combination with smooth muscle or striated muscle have been explanted. Smooth muscle from the uterus and striated muscle from the thigh of normal guinea pigs have been used. Prior to explantation, the infectious tissues and normal muscle have been incubated together at room temperature for at least 1 hour. The cultures have been maintained at 32–35°C. over a period of from 2 to 4 weeks. Transfers have been made at intervals of from 5 to 14 days. After termination of the period of cultivation, cultures have been selected for tests of infectivity. The remainder have been treated by the Regaud-Giemsa technique. Serial sections have been made of the tissues cultivated in plasma clots, as well as those in Maitland's medium.

Inasmuch as the cultures in plasma clots have been more successful than those in the fluid medium, the descriptions principally will be restricted to the former. They always have been infectious for

guinea pigs during the period of cultivation (2 to 4 weeks). There have been no important variations in the microscopic findings which are attributable to prolongation of the period of cultivation beyond 2 weeks.

The cultures of scrotal sac exudate after growth *in vitro* for 2 weeks have contained a few typical macrophages and rare polymorphonuclear leucocytes. The majority of the cells have been large mesenchymal cells, frequently in mitosis. These large fusiform or stellate cells have multiple processes, a reticulated cytoplasm, and prominent ellipsoidal nuclei.

In the average culture rickettsiae have been present in 80 to 90 per cent of the large mesenchymal cells and macrophages. The nuclei of approximately 20 per cent of the cells have been partially or completely filled with rickettsiae. Nuclei undergoing mitosis often have contained the microorganisms.

The characteristic, lanceolate, diplococcoid or diplobacillary forms of rickettsiae have been encountered most frequently. Isolated, coccoid or linear, bacillary forms have shown, as in the smears, the tendency of the organisms to be pleomorphic. Long chains have been found in many cells.

There has been a considerable range in the dimensions of the organisms. This has been noted within individual cells, among separate cells in the same culture, and among the different cultures. The range in size has occasionally exceeded the limits of that described in smears and sections of scrotal sac exudate previous to cultivation *in vitro*.

The intracytoplasmic distribution usually has conformed to one or two general patterns. A common localization has been in the reticulum between unstained spherical spaces which commonly occur in the cytoplasm of cells cultivated *in vitro*. These particular spaces represent the site of dissolved protoplasmic spherules which occur as highly refractile bodies in the living cell. It has been in this reticulum that the long chains of rickettsiae often have formed, although isolated diplococcoid structures have been most frequent. In the cells having no reticulum of the nature described above, there has been a random scattering of diplococci and a reduction in the number of chains. Occasionally the rickettsiae have been present in isolated clusters

surrounded by a narrow clear halo. These clusters frequently have been near the periphery of the cells and have never completely filled the entire cytoplasmic region.

The nuclei, although occasionally distended by a mass of rickettsiae, usually have contained small irregular clusters, one to four in number, centrally located and surrounded by a narrow clear halo. Short chains rarely have been distinguishable. The nucleoli and strands of chromatin have either disappeared or have been retained in scattered fragments among the rickettsiae or along the nuclear membrane. The nuclear membrane often has been thicker and more irregular than normal.

The intranuclear forms have varied greatly in size and shape. As a general rule, the more numerous the rickettsiae, the greater the number of small coccoid forms. There have been no sharp distinctions between the morphological range of the intranuclear forms and that of the intracytoplasmic rickettsiae. It has been a fairly uniform observation that in any single nucleus, all rickettsiae are very similar, while in the cytoplasm of a single cell, great morphologic variations have been found.

There has been no evidence that extensive intranuclear and intracytoplasmic parasitism is detrimental to the cell. On the contrary, rickettsiae, although generally in small numbers, have been found in the mitotic figures and regional protoplasm of dividing cells. It has been common, also, to find certain zones in growing cultures where intranuclear localization is much more prominent than elsewhere.

No additional useful data have been obtained from the cultures of splenic tissue. The significant findings have been identical with those in cultures of scrotal sac exudate.

Several attempts to infect uterine smooth muscle and striated muscle from the thigh have been unsuccessful. The serial sections have shown numerous rickettsiae in cells which had grown between the viable muscle fibers and into the protoplasm of degenerating muscle cells.

The cultures of tissues in Maitland's medium have shown multiplication of rickettsiae. The smears and serial sections of tissue cultivated in this manner have given less uniform and less satisfactory

data than the tissue cultivated in plasma clots, although very heavily infected cultures have been frequently obtained within a period of 2 weeks. As yet it has been impossible to determine whether there is an extracellular proliferation of the microorganisms in the Maitland medium but the weight of the evidence is to the contrary.

Analysis of Data

In this analysis, reference will be made to diseases of the typhus and spotted fever groups. The diseases of the typhus group include epidemic (classical) typhus and endemic (murine) typhus. The diseases of the spotted fever group include Rocky Mountain spotted fever, Eastern spotted fever, São Paulo typhus, Reimann's Minnesota disease, and possibly tick bite fever of South Africa. Our purpose is to inquire into the relationship of *fièvre boutonneuse* to these two groups of diseases.

Past experience has proved that in man, typhus fever cannot always be differentiated from spotted fever by clinical observations. However, two clinical facts of differential value have been derived from a consideration of *fièvre boutonneuse*. First, the disease often has followed the bite of a tick and an initial lesion frequently has formed at the site of the tick bite. Second, the cutaneous rash has characteristically involved the palms of the hands and the soles of the feet.

Although the tick may serve as the vector of several diseases, numerous varieties of the tick harbor the rickettsiae of all established diseases of the spotted fever group. The vectors of the typhus fevers are the flea and the louse. The problem of apparent vectorial specificity was approached by Zinsser and Castaneda (20). They proved that the typhus virus remained viable in the tick for as long as 14 days but introduced no evidence that the cells of the tick were invaded by the typhus rickettsiae or that the disease was transmissible by the bite of the tick.

The local inflammation in the region of the bite of the vector is not a general characteristic of the spotted fevers. Tick bite fever of South Africa, an assumed variety of spotted fever, often has a local lesion. The mite-borne rickettsial diseases usually have a similar but more severe reaction at the site of the bite of the vector. The exact

relationship between the mite-borne diseases and other rickettsial diseases has not been determined. No local cutaneous lesion follows the bite of the flea or louse, vectors of the typhus rickettsiae.

The distribution of the rash over the palms of the hands and the soles of the feet is characteristic of the spotted fevers and not of diseases of the typhus group.

The results of the Weil-Felix reaction in *fièvre boutonneuse* conform with those obtained in all but one disease of the spotted fever group. The exception is São Paulo typhus. The negative low titre agglutination of certain strains of *B. proteus* in the presence of serum from patients with *fièvre boutonneuse* is in direct contrast to positive high titre agglutinations which are typical of the typhus fevers.

The experiments concerned with the effect of convalescent sera on the virulence of the rickettsiae of *fièvre boutonneuse* also emphasize the absence of an immunologic relationship between *fièvre boutonneuse* and the typhus fevers.

The clinical aspects of *fièvre boutonneuse* in the guinea pig are compatible with those produced by certain strains, either of typhus fever or spotted fever. This is not surprising because clinical differentiation of these two groups of diseases in guinea pigs often is impossible.

The histopathology of guinea pigs with *fièvre boutonneuse* is characterized by small focal necroses, an acute thromboangitis, and the absence of cerebral lesions. The focal necroses and the severe acute angitis with accompanying fibrinous thrombi are always found in diseases of the spotted fever group. In the typhus fevers a mild angitis, insufficient to provoke the formation of fibrinous thrombi, is a constant finding and no focal necroses are demonstrable. The absence of cerebral vascular lesions favors a diagnosis of spotted fever, but no major significance is attached to either the presence or absence of such lesions.

The histopathologic study of human tissues is not sufficiently complete to warrant comment. The perivascular cellular infiltration in the subcutaneous tissues in *fièvre boutonneuse* is compatible with either the spotted fevers or the typhus fevers.

The tests for crossed immunity between *fièvre boutonneuse* and endemic as well as classical typhus are negative. Except for the

results of one investigator, all evidence indicates that there is a positive crossed immunity between *fièvre boutonneuse* and at least two diseases of the spotted fever group, namely Rocky Mountain spotted fever and São Paulo typhus. If the tests for crossed immunity had been inconclusive, as was true in the instance of Reimann's strain (7), we would have evaluated the evidence obtained by application of the other methods and drawn conclusions accordingly. In dealing with the puzzling atypical strains, this method of procedure is necessary, and had it been generally used in the past many errors, made especially by European investigators, would never have occurred.

The fact that Rocky Mountain spotted fever vaccine, prepared from ticks, does not protect guinea pigs against *fièvre boutonneuse* is of great interest. Further investigation of this problem is indicated because of its possible bearing on the cause of obvious differences which exist among various strains of rickettsiae in the same species.

The comparison of the morphology of the rickettsiae of *fièvre boutonneuse* with other pathogenic rickettsiae leads to definite conclusions. The microorganisms of *fièvre boutonneuse* characteristically occur in pairs. They are usually deeply stained with Giemsa's fluid and are often surrounded by a narrow clear halo. The typical form is lanceolate, although coccoid and linear bacillary forms may be found. There is a relatively great range in size but all gradations can be traced. These characteristics are typical of the rickettsiae of the spotted fevers. In contrast, the rickettsiae of the typhus fevers are delicate, linear, bacillary microorganisms which stain lightly with Giemsa and have only a comparatively small range in size. The common features of the rickettsiae of typhus and spotted fever are exemplified by the microorganisms of *fièvre boutonneuse*, namely the tendency to occur in pairs and to form chains.

The distribution of the rickettsiae of *fièvre boutonneuse* conforms with that of the several established strains of spotted fever. The distribution, as described in the guinea pig, in the *in vitro* cultures of infectious tissues, and in the vector differs greatly from that which is characteristic of the rickettsiae of the typhus fevers. These differences in localization are concerned not only with the types of cells which serve as a satisfactory environment for the specific microorganism but also the absolute partition of intracellular distribution.

The rickettsiae of fièvre boutonneuse and of the spotted fever group of diseases in guinea pigs are found in peritoneal serosal cells, macrophages, endothelial lining cells of blood vessels, smooth muscle cells of blood vessels, and in polymorphonuclear leucocytes (in the latter cell probably only by virtue of phagocytosis). In guinea pigs the rickettsiae of the typhus fevers are restricted to peritoneal serosal cells, endothelial cells of blood vessels, and polymorphonuclear leucocytes. Comparative studies by the method of tissue culture serve to emphasize these constant differences.

The rickettsiae of fièvre boutonneuse in the vector may be distributed throughout the viscera so as to be demonstrable in almost every type of cell. This diffuse parasitization is similar to that described by Wolbach (6) in ticks harboring the rickettsiae of Rocky Mountain spotted fever. This distribution is in contrast to the restricted localization of typhus rickettsiae to the intestinal lining cells of the flea or louse.

In concluding the analysis of data, let us consider the intracellular distribution of the rickettsiae of fièvre boutonneuse and compare it with the findings obtained by the study of several strains of spotted fever and typhus fever. The pattern of intracytoplasmic distribution and the presence or absence of intranuclear localization form the major subjects for inquiry.

The behavior of the rickettsiae of fièvre boutonneuse in the cytoplasm of cells is similar to that which is characteristic of the several varieties of spotted fever. This generalization applies equally to the cells of the vector and the infected guinea pig. The typical pattern is one of a random scattering of the microorganisms throughout the cytoplasm. When an intracytoplasmic reticulum is visible they usually follow its configuration. Occasionally they occur in small compact clusters. They never multiply within the cytoplasm to such an extent as to distend the cellular membrane in the manner which is typical of the rickettsiae of the typhus fevers. These characteristics become more definite after *in vitro* cultivation of infectious mammalian tissues and the refeeding of parasitized ticks.

Of greater significance than the intracytoplasmic distribution is the presence of rickettsiae within the nuclei of cells. This unique localization is a characteristic of the rickettsiae of the spotted fever group of

diseases and is never observed in typhus. It is a characteristic which is not shared by any microorganisms in mammalian tissues, if the so called filterable viruses may wholly be excluded from the category of intranuclear organisms. Intranuclear rickettsiae may be demonstrated in certain cells of the infectious scrotal sac exudate or spleen of the guinea pig and in various cells of the arthropod vector.

Fièvre boutonneuse is the first rickettsial disease in which intranuclear parasitism of mammalian cells has been demonstrable prior to the cultivation of tissues. Reimann's Minnesota disease (7), Eastern spotted fever (7), and Rocky Mountain spotted fever² have required the application of tissue culture methods before the localization is apparent.

The assumption that the rickettsiae within the nuclei of cells of the arthropod vector are representative of the pathogenic microorganisms causing fièvre boutonneuse may be severely criticized. The experiments were not conducted with sufficient foresight to permit a correlation of infectivity of individual ticks with the presence of intranuclear rickettsiae. Also the possibility that the ticks harbored more than one type of pathogenic rickettsiae was not excluded. Finally, intranuclear rickettsiae were found in only four ticks of the refed group and in none of the group not refed. In an attempt to justify our assumption we cite the following facts.

First, at least one of the fourteen ticks of the refed group contained pathogenic rickettsiae as established by the fact that the guinea pig upon which they fed acquired an immunity to fièvre boutonneuse. Second, refeeding is often essential before intranuclear rickettsiae may be demonstrated in ticks harboring the rickettsiae of Rocky Mountain spotted fever. Third, the diffuse invasion of the viscera of the four ticks in which intranuclear forms were found with rickettsiae indistinguishable from those in the scrotal sac exudate of guinea pigs with fièvre boutonneuse was prominent. Fourth, intranuclear microorganisms may not always be demonstrable, even under ideal conditions. Fifth, intranuclear rickettsiae have never been found in ticks which harbor exclusively nonpathogenic microorganisms.

Even though the generalization is made that the rickettsiae of the

² Hass, G. M., and Pinkerton, H., unpublished experiments.

spotted fevers and fièvre boutonneuse inhabit the nuclei of cells in ticks which serve as vectors, it would be unjustifiable to draw any conclusions of differential value by emphasizing the absence of intranuclear rickettsiae in the flea and louse which serve as the vectors of the typhus fevers. In order to dispense with the obvious errors which might arise from such a comparison, it has been expedient to study in tissue cultures a single type of mammalian cell, susceptible to parasitism by both genera of pathogenic rickettsiae. Thus the interior of this cell is utilized as a common culture medium. The cell so converted to this use is a large mesenchymal cell. In this cell the typhus rickettsiae, regardless of the variety or virulence, multiply freely in the cytoplasm. In this same type of cell under identical conditions, accurately controlled *in vitro*, the rickettsiae of several diseases of the spotted fever group and of fièvre boutonneuse proliferate, not only in the cytoplasm but also in the nucleus. The selective localization and typical distribution in each instance appear as a fair reproduction of the behavior of the rickettsiae in the cells of their respective vectors. Within the limits of our present methods of study this represents an external manifestation of natural traits and clearly separates the rickettsiae of the typhus fevers from those of the spotted fevers.

DISCUSSION

Our thesis is that a general solution of any rickettsial disease is to be sought at the focus of several methods of approach. These methods cannot be upheld as absolute or even sufficient. They have the advantage of being simple, of being subject to experimental test, and of yielding results which upon analysis give data which are moderately precise.

These methods have been applied to the study of many strains of rickettsiae. An analysis of the results thus far has given no justification for the recognition of more or less than two genera of pathogenic rickettsiae (each genus being composed of a single species), and two corresponding groups of rickettsial diseases in man. One genus and species (*Rickettsiae prowazeki*) includes the microorganisms which cause diseases of the typhus fever group. The other genus and species (*Dermacentroxenus rickettsi*) is composed of the rickettsiae of the various members of the spotted fever group. All evidence indicates

that fièvre boutonneuse is a variety of spotted fever and that its etiologic agent belongs to the corresponding genus and species.

With these gross subdivisions as a foundation, some inquiries into the cause of slight variations among the strains of each established genus and species (such as the above mentioned immunological differences found by Parker to exist between the rickettsiae of fièvre boutonneuse and Rocky Mountain spotted fever) may be made in an orderly manner. Such variations, although of subspecific value, are real, and in general have evaded satisfactory explanation.

CONCLUSIONS

Several methods for the experimental study of the rickettsial diseases have been applied to fièvre boutonneuse. An analysis of the results has indicated that fièvre boutonneuse is a variety of spotted fever and that the etiologic agent is a rickettsia which belongs to the genus *Dermacentroxenus* and to the species *rickettsi* (Wolbach (6)). The distinctive morphology of the organism and its characteristic intranuclear clustering in ticks and in tissue cultures are the important criteria upon which this conclusion is based. Immunological, histological, and cytological observations of a confirmatory nature are also reported.

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EXPLANATION OF PLATES

PLATE 35

FIG. 1. Photomicrograph of proliferating mesenchymal cells derived from scrotal sac exudate which had been cultivated *in vitro* for 2 weeks. The region within the nuclear membrane of one cell is largely occupied by rickettsiae and there is a random distribution of the microorganisms throughout the cytoplasm of all cells. Regaud fixation. Giemsa stain. $\times 2600$.

FIG. 2. Photomicrograph of cells of scrotal sac exudate which had been cultivated *in vitro* for 3 weeks. The nucleus of one cell contains a mass of rickettsiae. The cytoplasm of the adjacent cell shows the maximum degree of parasitization. Regaud fixation. Giemsa stain. $\times 2800$.

FIG. 3. Photomicrograph of a mesenchymal cell which has grown from the margin of explanted scrotal sac exudate, cultivated *in vitro* for 3 weeks. The nucleus of this cell shows the typical central aggregation of rickettsiae and the random cytoplasmic distribution. Regaud fixation. Giemsa stain. $\times 2600$.

FIG. 4. Photomicrograph of a mesenchymal cell interpreted as a macrophage, derived from scrotal sac exudate cultivated for 2 weeks *in vitro*. Note the accumulation of rickettsiae in the center of the nucleus, the halo around the mass of microorganisms, and the isolated coccoid forms in the cytoplasm. Regaud fixation. Giemsa stain. $\times 3300$.

PLATE 36

FIG. 5. Photomicrograph of a segment of a Malpighian tubule of a tick (*Rhipicephalus sanguineus*) parasitized with rickettsiae of fièvre boutonneuse. One nucleus is distended with rickettsiae. There are a few indistinct organisms in the cytoplasm of each cell. Regaud fixation. Giemsa stain. $\times 3400$.

FIG. 6. Photomicrograph of an artery in the polar fat of the testis of a guinea pig with fièvre boutonneuse. The entire thickness of the vascular wall has been involved in the inflammatory reaction and a mural thrombus has formed. Regaud fixation. Hematoxylin-eosin stain. $\times 275$.

PLATE 37

FIG. 7. Camera lucida drawing of the rickettsiae of fièvre boutonneuse in the endothelial cells of the intima and smooth muscle cells of the media of a venule in the polar fat of the testis of a guinea pig. Regaud fixation. Giemsa stain. $\times 2000$.

FIG. 8. Camera lucida drawings $\times 2500$. Regaud fixation. Giemsa stain.

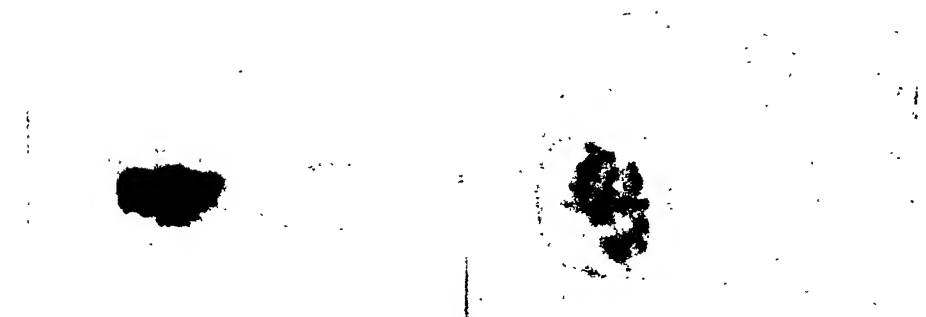
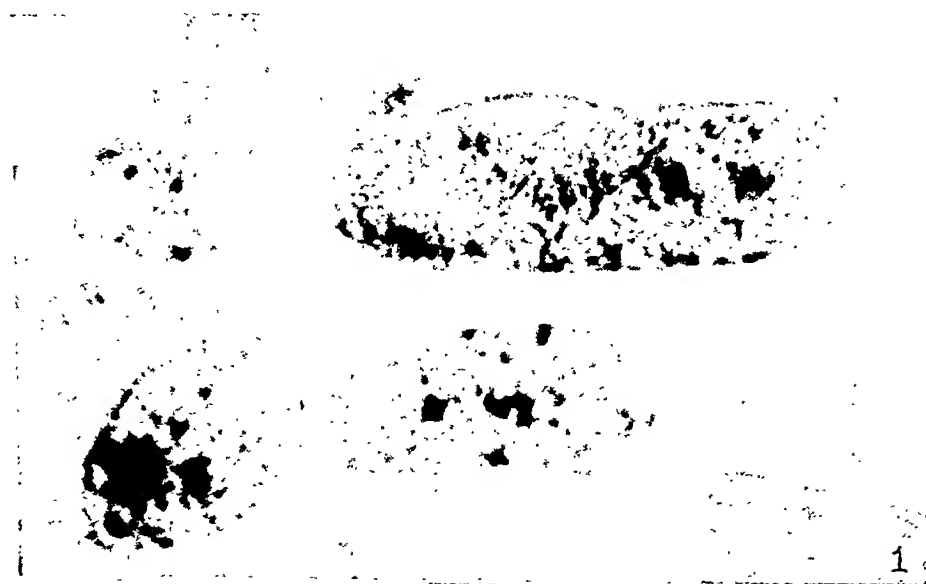
The upper left hand cell shows rickettsiae of fièvre boutonneuse in the nucleus and cytoplasm. The cell was selected for illustration from scrotal sac exudate cultivated *in vitro* for 2 weeks. This type of localization is typical of diseases of the spotted fever group.

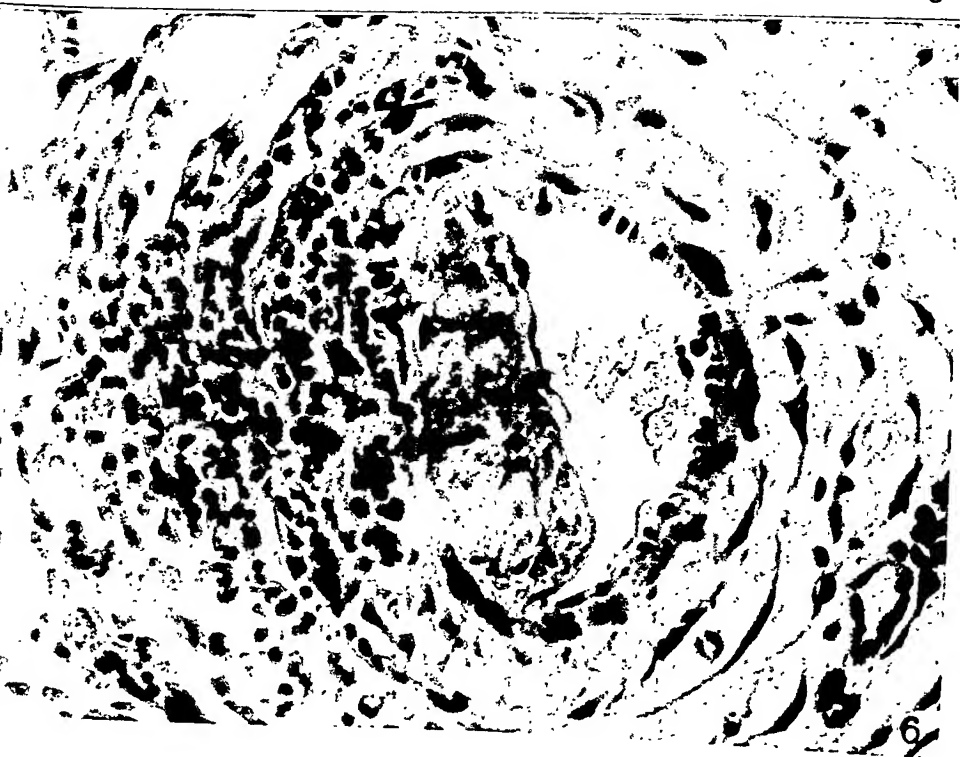
The upper right hand figure shows rickettsiae of endemic typhus in the cytoplasm of a cell at the proliferating margin of scrotal sac exudate cultivated *in vitro* for 2 weeks. The localization and degree of intracellular growth are characteristic of diseases of the typhus group.

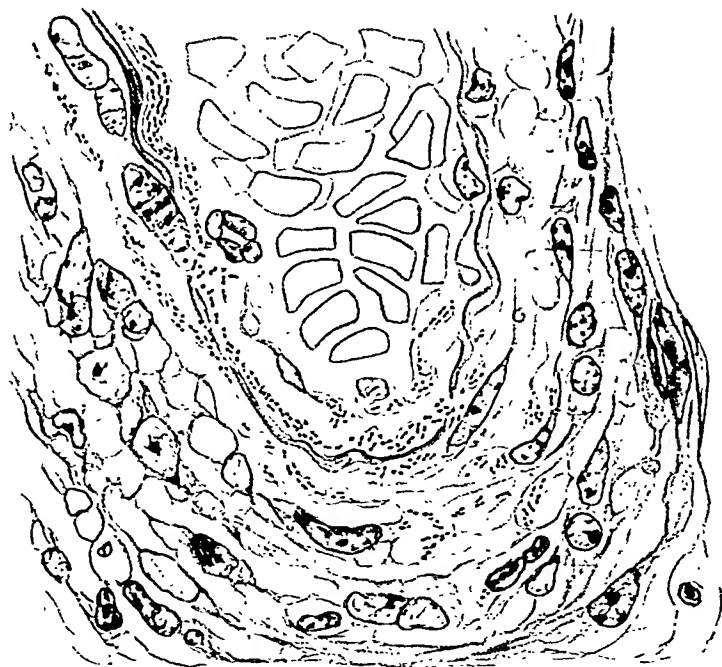
The lower left hand figure is a drawing of a cell from the hypoderm of a tick (*Rhipicephalus sanguineus*) harboring rickettsiae of fièvre boutonneuse. Note the intranuclear and intracytoplasmic parasitism.

The lower right hand cell is from the lining of the gut of a louse infected with rickettsiae of European typhus. The same type of localization of rickettsiae is found in fleas infected with typhus.

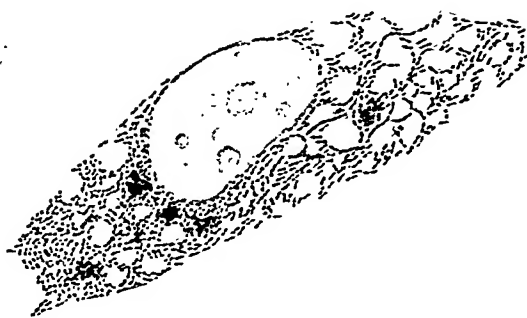
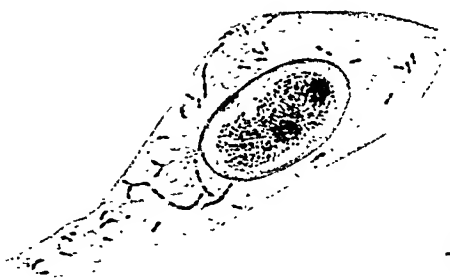
Compare the patterns of intracellular parasitism. Note that the rickettsiae of fièvre boutonneuse exhibit characteristics which distinguish them from those of typhus fever and which ally them closely with those of the spotted fevers.







7



STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS. II

By K. LANDSTEINER, M.D., AND JOHN JACOBS, M.D.

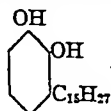
(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 10, 1936)

In an earlier paper (1) experiments were described in which, with the use of a suitable method, sensitization effects were produced by numerous simple compounds with satisfactory regularity. The work has been continued and also extended to include a number of substances not studied hitherto.¹

Urushiol and Related Substances.—Urushiol is a substance contained in Japanese lacquer from *Rhus vernicifera*² which, like poison ivy, *Rhus toxicodendron*, produces intense dermatitis in a certain percentage of individuals.

The chemical identity of the effective substance in *Rhus toxicodendron* has been the subject of controversy, various substances having been held responsible. Acree and Syme (2) believed it to be a complex glycoside, and by others the active principle has loosely been described as an oleoresin. But a series of studies carried out by Majima (3), on the chief constituent of *Rhus vernicifera*, led him to the isolation of urushiol, a pale yellow viscous oil boiling at 200–210°C. at 0.4–0.6 mm., soluble in alcohol and petrol ether, which he showed to be chiefly *o*-dihydroxybenzene with a straight, unsaturated fatty chain, of the formula



According to Majima his preparation was not homogeneous but contained substances differing in the number and position of double bonds in the side chain, and also hydrourushiol. Urushiol was found by Toyama (4) and Majima (3) to

¹ The statement about negative results with quinine may need correction because one apparently positive and some questionable results have since been obtained in treated animals.

² We wish to express our thanks to the Mitsui Co. for their kind cooperation in furnishing a supply of Japanese lacquer.

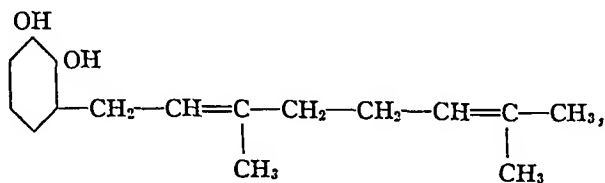
be the substance responsible for the injurious effects in human beings. In agreement with these findings McNair (5) reported that the toxic substance in poison oak, *Rhus diversiloba*, is probably an unsaturated ortho-dihydroxybenzene derivative, and later Hill and his coworkers (6) identified the active agent of poison ivy with that in Japanese lacquer, namely urushiol.

Sensitization of guinea pigs to poison ivy extracts, but not to the isolated active substance has been described by Rackemann and Simon (7), Dienes (8), and Simon (9). Therefore, the following experiments were made on guinea pigs with urushiol and related compounds.

One drop of a 3.0 per cent acetone solution of distilled urushiol was spread on the back (the hair having been clipped) with a glass rod, twice a week for 2 weeks, and tests were made after an interval of 3 weeks by gently spreading one drop of a 0.3 per cent acetone solution of urushiol on the flank; the reactions were recorded after application of a depilatory.

Distinct, pinkish reactions appeared at the sites of application by the next morning. On cross testing these animals and guinea pigs similarly sensitized to poison ivy extract, positive reactions were observed.

In order to study the influence of variations in molecular structure on sensitizations with this sort of substance, a related compound, geranyl catechol,



was synthesized according to the method of Kawai (10), and animals were sensitized as with urushiol excepting that an 0.8 per cent solution in acetone was used for testing. Some of the animals showed hypersensitiveness. In cross tests between geranyl catechol and urushiol overlapping reactions were observed when animals treated with urushiol were tested with geranyl catechol.

With another compound, 4-tetradecyl catechol, prepared from myristic acid and catechol (11), some evidence of sensitization, although slight, was obtained in a limited number of guinea pigs, and

a few animals sensitized to urushiol gave faint reactions when treated with a 2.0 per cent solution of this compound in olive oil.

The biological activity of urushiol may well be connected with its oxidizability; observations by Hill (6) and Toyama (4) would tend to show that methylation of the hydroxyl groups greatly diminishes activity, and hydrogenation of the double bonds in the side chain considerably reduces it, according to the latter author; Hill found only a slight diminution.³

Methyl Heptene Carbonate $\text{CH}_3(\text{CH}_2)_4\text{C} \equiv \text{C}.\text{CO}_2\text{CH}_3$.—Another product examined was a commercial preparation, methyl heptene

TABLE I*

Application of a 20 Per Cent Solution in Alcohol of Methyl Heptene Carbonate on the Skin

No.	Animals sensitized to methyl heptene carbonate	No.	Controls
1	p., m.el.	7	a.neg.
2	d.p., sl.el.	8	a.neg.
3	p., sl.el.	9	f.-p.p.
4	p.p.-p., sl.el.		
5	p., sl.el.		
6	p., sl.el.		

* Here and in subsequent tables the following abbreviations are used: almost colorless (a.cls.), faintly pink (f.p.), pale pink (p.p.), pink (p.), and dark pink (d.p.). Other designations are: negative (neg.), almost negative (a.neg.), slightly elevated (sl.el.), elevated (el.), markedly elevated (m.el.), slightly swollen (sl.swol.), and swollen (swol.), livid center (liv.c.), necrosis (necr.).

carbonate, which was investigated because several cases have been described (12, 13) of hypersensitiveness to this substance in human beings when cosmetics were used.⁴ The substance seemed of interest because it is an aliphatic compound, containing no aromatic rings.

Ten intracutaneous injections of 1/20 mg. in 0.05 cc. olive oil on the back, at weekly intervals, were followed by 2 weeks of rest, and the animals were tested by gently spreading 1 drop of a 20 per cent alcoholic solution of the substance on the flank.

³ Concerning the toxicity of other catechol derivatives see Toyama (4).

⁴ A preliminary report has appeared (14).

It may be seen in Table I that, although controls were negative, or, in a few cases, faintly pinkish, many treated animals gave frankly pink, raised lesions, indubitable evidence of sensitization, which was confirmed by a number of specificity tests.

TABLE II

Substance	K^*	Reaction with aniline	Sensitization
1:2:4 Chlorodinitrobenzene.....	0.110 (0°); 3.26 (15°, eth.)	+	pos.
1:2:4 Bromodinitrobenzene.....	1.89 (15°, eth.)	+	"
1:2:4 Iododinitrobenzene.....	0.455 (15°, eth.)	+	"
1:2:4 Fluorodinitrobenzene.....	686.0 (15°)	+	"
1:4:2:6 Dichlorodinitrobenzene.....	0.0248 (0°)	+	"
1:3:4:6 Dichlorodinitrobenzene.....	1.20 (0°)	+	"
1:2:4:6 Chlorotrinitrobenzene.....	Very great† (0°)	+	"
1:3:5 Dichloronitrobenzene.....	Reaction irregular‡	—	neg.
1:4:2 Dichloronitrobenzene.....	0.00000297 (0°)	—	"
1:2:4 Dichloronitrobenzene.....	0.0000183 (0°)	—	"
<i>p</i> -Chloronitrobenzene.....	0.000000987 (0°)	—	"
<i>p</i> -Dichlorobenzene.....	0.00019 (175°)	—	"
1:2:4 Trichlorobenzene.....	Very small§ (0°)	—	"
1:2:4:5 Tetrachlorobenzene.....	Very small§ (0°)	—	"
Hexachlorobenzene.....	Very small (175°)	—	"

* Constants for the velocity of decomposition of the substances by sodium methylate, or sodium ethylate (eth.) at the temperature indicated. The figures are taken from papers from the Laboratory of Organic Chemistry of the University in Amsterdam (*Rec. trav. chim. Pay-bas*, 1890-1924, Vols. 9 to 43), mostly by Holleman and coworkers.

† Velocity too great to be measured.

‡ Formation of azoxy compounds.

§ Value found by the authors to be very small, less than or of the same order as that for 1:2:4 dichloronitrobenzene.

On repeating the tests, it was found that different samples of the commercial substance showed distinct gradations in activity. It is therefore possible that the effect is due, not to methyl heptene carbonate itself, but to some impurity. In fact, on account of the method of preparation, one may assume that it is not very easy to obtain the substance in a condition of absolute purity. (The preparations used did not contain nitrogen.)

Cl, NO₂ Substituted Benzenes.—Our former results suggested strongly that ability to sensitize is connected with the chemical reactivity of the substances, making it probable that they form compounds in the animal body possessing antigenic activity. Table II, presenting more detailed data, will serve to support this conclusion. The table gives the sensitizing capacity of the chloro- and nitro- substitution products of benzene tested, along with the constants calculated from the reaction with sodium methylate, indicating the rate at which Cl is split off by alkali. One sees that those substances which have a very low constant did not sensitize, and also that the lowest constant of an active substance was many times greater than the highest constant of any inactive substance.

TABLE III

Substance	K_0^*	Reaction with aniline	Sensitization
1:2:4:5 Dichlorodinitrobenzene.....	0.326		
1:3:2:5 Dichlorodinitrobenzene.....	0.145	+	pos.
1:2:4 Trinitrobenzene.....	Very great†	+	"
1:3:5 Trinitrobenzene.....	1.57	+	"
<i>m</i> -Dinitrobenzene.....	Reaction irregular‡	—	neg.
		—	"

* Constants for the velocity of decomposition of the substances at 0°C. by sodium methylate.

† Velocity too great to be measured.

‡ Formation of azoxy compound.

Turning now to those compounds in which the NO₂ group is replaced on treatment with sodium alcoholate or which do not contain chlorine, namely 1:2:4 and 1:3:5 trinitrobenzene, 1:2:4:5 and 1:3:2:5 dichlorodinitrobenzene, and *m*-dinitrobenzene, one finds that the correlation does not hold in that 1:3:5 trinitrobenzene ($K_0 = 1.57$) had no sensitizing capacity whereas the two dichlorodinitrobenzenes ($K_0 = 0.1$ and 0.3) and some chlorine labile compounds with smaller constants, were effective (Table III).

On account of this discrepancy we investigated how the substances react with an organic base (aniline), and the same sort of experiment was made with the compounds listed in Table III.

1. The substances known to possess labile halogen (Table II) were dissolved in absolute alcohol and aniline (5 mols) added. The mixtures were heated in sealed tubes in the steam bath for 2 hours and the quantity of liberated halogen determined. In column 3 of Table II the symbol + designates almost complete liberation of halogen (more than 90 per cent) and the symbol - indicates that no or very little (less than 5 per cent) halogen was replaced.

2. Five millimols of the substances appearing in Table III were dissolved in 10 cc. of absolute alcohol and 15 millimols of aniline added. The mixtures were heated in sealed tubes in the steam bath for 15 hours, evaporated to dryness, washed with a little absolute alcohol, and recrystallized twice from the same solvent. (The same results were obtained on heating mixtures set up as in (1) for 2 hours). Column 3 of Table III summarizes the experiments, + being used to indicate the formation of a substitution compound, and - for addition compound formation.

Now it was found that 1:2:4 trinitrobenzene, 1:2:4:5 and 1:3:2:5 dichlorodinitrobenzene gave substitution compounds with aniline whereas this was not the case with 1:3:5 trinitrobenzene and *m*-dinitrobenzene.

The compound formed on treating 1:2:4 trinitrobenzene with aniline has been described by Hepp (15); from 1:2:4:5 dichlorodinitrobenzene we obtained an orange colored substance melting at 94–95°C., N calculated 9.89 per cent (for $C_{12}H_8O_2N_2Cl_2$), found 9.83 per cent; from 1:3:2:5 dichlorodinitrobenzene a compound melting at 114–115°C., N calculated 9.89 per cent (for $C_{12}H_8O_2N_2Cl_2$), found 9.71 per cent. 1:3:5 trinitrobenzene forms a highly colored addition compound (15) which gives off aniline readily when exposed to air; *m*-dinitrobenzene also forms an addition compound (16), and in both cases the original nitro compound was readily recovered unchanged.

Of the substances given in Table II those which sensitized, formed substitution compounds; in all these cases 90–100 per cent of the theoretical amount of halogen was liberated. The nonsensitizing substances treated in the same way gave off no or but little chlorine.

The difference in sensitizing activity of 1:2:4 trinitrobenzene (active) and 1:3:5 trinitrobenzene (inactive) mentioned before, but without details, was reinvestigated with a larger number of animals, since it seemed to be particularly striking on account of the structural similarity of the two compounds, different only in the position of the NO_2 groups. Parallel experiments with the two compounds were carried out as given below.

In both cases, a batch of white guinea pigs was injected with 1/400 mg. (0.1 cc. of a solution made by adding 0.5 cc. of a 0.3 per cent alcoholic solution to 60 cc. saline) intracutaneously, on the back, at weekly intervals for 10 weeks and after 2 weeks of rest the animals were tested by gently spreading 1 drop of a 1 per cent olive oil solution, or by an injection similar to those used for sensitization, on the flank. The first mode of application, namely to the surface of the skin, gave the most striking effects, the controls being, as a rule, com-

TABLE IV

Treated and tested with	Treated animals			Controls		
	No.	Application of a 1 per cent solution in olive oil on the skin	Intracutaneous injection of 1/400 mg. in 0.1 cc. saline	No.	Application of a 1 per cent solution in olive oil on the skin	Intracutaneous injection of 1/400 mg. in 0.1 cc. saline
1:2:4 Trinitrobenzene	10	f.p.	8,* p.p., el.	21	neg.	neg.
	11	p., el.	10, f.p., el., liv.c. 3	22	"	5, p.p., el.
	12	f.p.	9, p.p., el.	23	"	neg.
	13	p.p.	8, p., el.	24	"	a.neg.
	14	p., sl.el.	9, p.p., el.			
1:3:5 Trinitrobenzene	15	neg.	neg.	25	a.neg.	neg.
	16	"	"	26	neg.	a.neg.
	17	"	"	27	"	a.neg.
	18	"	"	28	a.neg.	neg.
	19	"	"			
	20	"	a. neg.			

* The figures give diameters of the lesions in millimeters.

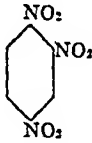
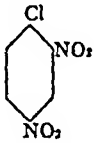
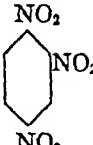
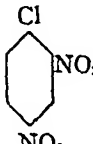
pletely negative. The results show that the 1:2:4 substituted compound is an active sensitizing agent, producing lesions similar to those obtained with 2:4 dinitrochlorobenzene (1), while the symmetrical compound proved to be entirely inactive (Table IV).

The above comparison of the substances demonstrates a parallelism, in the compounds examined so far, between sensitizing capacity and chemical behavior. In most cases a correspondence was evinced between lability of Cl and NO₂, when treated with alkali, and sensitizing effects; and in all cases tested there was agreement between

ability to sensitize and formation of substitution compounds with an organic base. It seems reasonable to assume that in the animal a reaction takes place by which the substances are converted into antigens. How and with what substances, proteins or others, a combination of the active compounds occurs, remains to be ascertained. For some of the sensitizers dealt with herein, compounds with amino acids or peptides have been reported (17, 18); also with thiols (19, 20) and phenols (21).

TABLE V

Application of One Drop Each of a 2.0 Per Cent Solution of 1:2:4 Trinitrobenzene and 1.0 Per Cent of 2:4 Dinitrochlorobenzene in Olive Oil on the Skin

Treated with	No.	Tested with	
			
	29	p., el.	p., el.
	30	f.p.	f.p.
	31	p., el.	p., el.
	32	p.p.-p., el.	p.p.-p., el.
	33	f.p.	f.p.
	34	p., el.	p., el.
	35	p., el.	p., el.
	36	p.p., el.	p., el.
Controls	37	neg.	neg.
	38	"	"
	39	"	"

In keeping with the foregoing was the fact that the two substances 2:4 dinitrochlorobenzene and 1:2:4 trinitrobenzene gave overlapping reactions, as has already been noted (1); in fact, they were practically interchangeable, a result to be expected inasmuch as both compounds will, after removal of Cl or NO₂, respectively, yield the same conjugate (Table V).

Various ways to sensitize intracutaneously with 2:4 dinitrochlorobenzene⁵ have been tested simultaneously in batches of 5 animals each: 16 injections, 2 a week (the poorest, perhaps by chance); 16 daily injections; 8 weekly injections; and 8 injections, 2 each week; all of 1/400 mg.; also 16 rubbings, 2 a week, of a 1 per cent olive oil solution. The animals were tested 2 weeks in this experiment, after the last injection, before testing by application of a 1 per cent olive oil solution. All of the above treatments resulted in distinct sensitization although 8 weekly injections seemed to be somewhat the superior method, by a slight margin. At another time a very satisfactory effect was seen after 8 daily injections; the animals were tested after an interval of 25 days.

The striking superiority of cutaneous sensitization, already mentioned in our recent paper, had previously been emphasized by Sulzberger (23) who also reported observations on variations in efficiency of intracutaneous and other methods of administration, in ordinary anaphylaxis. Attempts to sensitize guinea pigs intravenously or subcutaneously to 2:4 dinitrochlorobenzene were almost ineffective in three experiments. However, since one experiment gave doubtful results, possibly due to faulty technique, further confirmation would be desirable.

Benzyl Chlorides.—From the point of view suggested, it should be possible to find substances producing sensitization merely on the basis of their chemical properties. Consequently, it appeared promising to examine benzyl and acyl chlorides, containing easily detachable chlorine, although these substances have not yet been reported as sensitizing agents in human beings.

The experiments on benzyl chlorides were in general similar in their outcome to those observed with the substances already discussed. Marked results were obtained with *o*-chlorobenzyl chloride and 2:4 dinitrobenzyl chloride, and positive effects were noted likewise with *p*-chlorobenzyl chloride, benzyl chloride, and *p*-nitrobenzyl chloride.

The solutions for injection were prepared as above. In each case 1/100 mg. was injected twice a week for 12 weeks, followed by 2 weeks of rest. Tests were made with the solution used for sensitization, or olive oil solutions, a 20 per cent solution being used for *o*-chlorobenzyl chloride, and a 1.0 per cent solution for 2:4 dinitrobenzyl chloride. The results with these two substances are summarized in Table VI.

⁵ Since our communications Wedroff and Dolgoff (22) have reported the experimental sensitization of human beings with the substance.

When 2:4 dinitrochlorobenzene was compared with 2:4 dinitrobenzyl chloride a distinct difference was noted although cross reactions were observed; 2:4 dinitrochlorobenzene was different from *o*-chlorobenzyl chloride (Table VII). The two benzyl chlorides, on the other hand—2:4 dinitrobenzyl chloride and *o*-chlorobenzyl chloride—interacted.

TABLE VI

Application of a 1 Per Cent Solution of 2:4 Dinitrobenzyl Chloride in Olive Oil on the Skin

No.	Animals sensitized to 2:4 dinitrobenzyl chloride	No.	Controls
40	p.	56	neg.
41	p.p.	57	"
42	d.p.	58	"
43	d.p.	59	"
44	p.p.-p.	60	"
45	p.p.	61	f.p.-p.p.
46	p.p.		
47	p.p.		

Application of a 20 Per Cent Solution of o-Chlorobenzyl Chloride in Olive Oil on the Skin

No.	Animals sensitized to <i>o</i> -chlorobenzyl chloride	No.	Controls
48	p.p., sl.el.	62	neg.
49	p., sl.el.	63	"
50	d.p., el.	64	a.neg.
51	p., sl. el.	65	a.neg.
52	p.p.	66	a.neg.
53	p., sl.el.		
54	p.p.-p., el.		
55	p., el.		

Acyl Chlorides.—In experiments made on a small scale, indications of sensitization were obtained with some acyl chlorides, such as benzoyl chloride, *p*-bromobenzenesulfonyl chloride, *m*-nitrobenzenesulfonyl chloride, and 2-nitrotoluene-4-sulfonyl chloride.

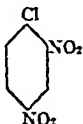
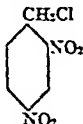
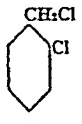
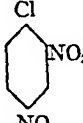
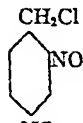

One of the acyl chlorides, *p*-chlorobenzoyl chloride, which exhibited unusually strong reactions, well developed after 24 hours, after the third or fourth injection of 1/80–1/100 mg. in olive oil solution, with

edema and pinkness over an area several centimeters in diameter and central necrosis, was investigated more extensively.

In such an experiment four weekly injections of 1/100 mg. *p*-chloro-benzoyl chloride in 0.05 cc. olive oil were given intracutaneously on the back, when necrotic lesions began to develop following new injections. The guinea pigs were then rested for 2 weeks and tested by spreading

TABLE VII

Combined Table. Application of One Drop of an Olive Oil Solution on the Skin

Treated with	No.	Treated with		
				
	67	f.p.	neg.	neg.
	68	p., el.	"	"
	69	p., el.	"	"
	70	p., el.	f.p.	"
	71	p., el.	neg.	"
	72	p.	p., sl.el.	p.
	73	neg.	p., sl.el.	f.-p.p.
	74	p.p.-p.	p.	p.p.
	75	f.p.	p.	f.-p.p.
	76	a.neg.	p.	f.p.
	77	neg.	f.p.	p.
	78	"	p.p.	p.
	79	"	f.-p.p.	p., el.
	80	"	f.p.	p.
	81	"	p.p.	p.

1 drop of a 10 per cent olive oil solution on the flank with a glass rod, or by an injection like those used for sensitization. In the surface applications, areas in contact with the olive oil solution developed pinkish to pink skin reactions of the usual appearance (Table VIII). Following injections with oil solutions the sensitized guinea pigs showed lesions similar to those observed during the treatment (Table IX).

TABLE VIII

No.	Skin reactions in sensitized animals after application of 10 per cent <i>p</i> -chlorobenzoyl chloride in olive oil	Amount injected	Intravenous injection of <i>p</i> -chlorobenzoylated guinea pig serum
		mg.	
82	p.p.	10	Typical anaphylaxis † 6 min.
83	p.p.-p.	5	" " † 5 "
84	p., sl.el.	2	" " † 5 "
85	p., el.	2	" " † 5 "
86	p.	2	" " † 7 "
87	p., sl.el.	1	" " † 15 "
88	p.p.	1	" " † 2 "
89	p.p., el.	1/4	" " † 6 "
90	p.p.	1/10	Coughs, eyes running; recovered
	Controls		Controls
91	neg.	10	No symptoms
92	"	10	" "
93	"	10	" "
94	"	2	" "
95	"	1	" "
96	"	1/4	" "
97	"	1/10	" "

† Death of animal.

TABLE IX

Intracutaneous Injection of 1/80 Mg. p-Chlorobenzoyl Chloride in 0.05 Cc. Olive Oil

No.	Animals sensitized to <i>p</i> -chlorobenzoyl chloride	No.	Controls
98	35,* p., swol., necr. 6	106	9, a.cls., m.el.
99	25, p.p.-p., swol., necr. 7	107	11, p.p., el.
100	40, p.p.-p., sl.swol., necr. 7	108	8, p.p., m.el.
101	40, p.p.-p., sl.swol., necr. 9	109	10, p.p., m.el.
102	45, p.p., swol.	110	9, p.p., el.
103	40, p.p.-p., sl.swol., necr. 8		
104	40, p.p.-p., sl. swol., necr. 8		
105	40, p.p.-p., sl.swol., necr. 6		

* The figures give diameters of the lesions in millimeters.

Animals sensitized to *o*-chlorobenzyl chloride were rather sharply distinguishable in their reactions from those treated with *p*-chlorobenzoyl chloride. A cross test with animals sensitized one each to the following substances:

p-bromobenzenesulfonyl chloride, *m*-nitrobenzenesulfonyl chloride, *p*-chlorobenzyl chloride, and benzyl chloride showed a marked degree of specificity except for cross reactions between the two sulfonyl chlorides.

Thus, while in discussions of the subject one encounters the opinion that there is no connection between the ability of substances to sensitize and their chemical nature, it appears from the foregoing that whole groups of substances—benzyl chlorides, acyl chlorides—characterized by a certain chemical reactivity, have the capacity to produce sensitization.⁶

With acyl chlorides there was the possibility of preparing protein compounds containing the acyl radicals and it was clearly of interest to investigate the effects of such conjugates in animals sensitized with the chlorides. This was done in the case of *p*-chlorobenzoyl chloride.

To 30 cc. of guinea pig serum (or horse serum) was added 15 cc. normal sodium carbonate solution and 1 millimol of *p*-chlorobenzoyl chloride in 5 cc. chloroform. The mixture was shaken vigorously for 10 minutes, centrifuged, the water layer acidified to maximum precipitation, centrifuged, and washed several times. The substance was redissolved in saline solution with alkali, neutralized, and made up to a 2 per cent solution.

When small quantities of the protein preparation were injected intravenously into guinea pigs sensitized to *p*-chlorobenzoyl chloride and showing positive skin reactions, a month after the skin tests, it was found that they died in acute, typical anaphylactic shock, as shown in Table VIII.

Injected into the skin, the acylated protein produces an immediate flare and wheal, followed, in stronger concentrations, by large pinkish edematous reactions on the next day.

The anaphylactic experiment described resembles to some extent the production of an anaphylactic state by injections of diazonium compounds (25) with the distinction, however, that in the latter case no skin reactions were observed of the type of "contact dermatitis" as is seen with the acyl chlorides.

This result, together with the before mentioned skin reactions with the substance, indicate that the two forms of hypersensitiveness observed in this case, sensitization of the skin and anaphylaxis, are

⁶ In this regard reference may be made also to the work of Mayer (24).

intimately associated states. Thus there may be produced in an animal by one and the same treatment an allergic state with the following manifestations: pink, sometimes elevated eruptions on the treated site resulting from superficial skin applications of the simple substance; large, pinkish, edematous lesions following its intracutaneous injection in small quantities; an immediately noticeable flare and swelling, developing into rather large lesions within a day, and typical anaphylaxis, on intracutaneous and intravenous injection, respectively, of the protein compound.

Benzoyl chloride behaved, in all respects, much like *p*-chlorobenzoyl chloride.

Further evidence relating to anaphylaxis induced by chemicals was afforded by experiments with arspenamine, to be reported later, in which typical anaphylactic shock was produced in a high proportion of the guinea pigs treated.

The chemical experiments were carried out with the assistance of Mr. Robert A. Harte.

SUMMARY

In continuation of previous work sensitization experiments have been made with various substances such as urushiol, benzyl chlorides, and acyl chlorides. In the case of a series of substituted benzenes (Cl, NO₂) a connection between sensitizing capacity and lability of the Cl or NO₂ groups has been shown, indicating the formation of conjugated antigens in the animal. This led to the study of benzyl and acyl chlorides which, actually, were found to have sensitizing capacity. Most informative as to the relationship between reactions of the skin surface and anaphylaxis were experiments with acyl chlorides. Guinea pigs sensitized with *p*-chlorobenzoyl chloride showed, on the one hand, the usual surface lesions after application of the substance, and on the other typical anaphylactic shock following intravenous injection of a compound of *p*-chlorobenzoyl chloride and guinea pig serum; from which it may be inferred that the two types of allergic manifestation are closely related conditions.

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THE RELATION BETWEEN ANTIANAPHYLAXIS AND ANTIBODY BALANCE*

I. THE RÔLE OF EXCESS OF CIRCULATING ANTIBODY IN HYPERSENSITIVENESS

By MARION C. MORRIS, Ph.D.

(From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis)

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At the present time the most widely accepted view concerning the mechanism of hypersensitiveness is that symptoms occur when antigen combines with fixed but not with circulating antibody, and that an excess of circulating antibody reduces the hypersensitive response of the sensitized animal by preventing access of antigen to sensitized tissues, or by causing it to reach the cells so slowly and gradually that no explosive reaction occurs.

The most direct evidence supporting such a view may be found in the work of Weil (1) who injected additional antibody into the circulation of guinea pigs already passively sensitized, and then tested the animals with antigen. He reported that such animals are actually protected against several lethal doses of antigen. Analogous observations have been made more recently by Dale and Kellaway (2) who found that the uterus of a sensitized guinea pig suspended in a bath to which antibody has been added will not contract when antigen is introduced, presumably because the latter is intercepted by the antibody in solution as it is intercepted in the living animal by circulating antibody before it can reach the sensitized cells. On the other hand, the work of Friedberger (3) indicated that such protection as was occasionally noted following the introduction of additional immune serum into sensitized animals was very slight and was not due directly to the antibody content of the antiserum but rather to traces of antigen remaining in it. Similarly, the work of von Fennyvessy and Freund (4) failed to offer any evidence of the protective effect of an excess of circulating antibody against anaphylactic shock.

Notwithstanding this meager amount of confirming evidence to support Weil's concept of the rôle of circulating antibody in anaphylaxis, his contributions have

*To Dr. J. J. Bronfenbrenner, who suggested these investigations, we are indebted for assistance, advice, and interest throughout the course of this study.

been largely responsible for the view that anaphylaxis is a purely cellular reaction, and that resistance to shock in a sensitized animal depends upon the presence of humoral antibodies. By analogy, Weil (5) applied this same concept to explain the mechanism of resistance to infection, also. Because of the confusion arising from such a view, the precise relation of the states of anaphylaxis, antianaphylaxis, and immunity to each other and to antibody balance is still somewhat unsettled. Before attempting to determine the relation of these various states to each other, it seemed essential to reinvestigate the rôle of circulating antibody in the hyper-sensitive animal.

Method

Guinea pigs of a uniform weight were passively sensitized by intraperitoneal injection of 0.5 cc. of rabbit antiserum. Following an incubation period of approximately 24 hours, the sensitized animals were divided into three groups. In the first, the lethal dose of antigen was determined by intravenous injection. In the second group of animals, an excess of antibody was given intravenously 20 to 60 minutes preceding the intravenous injection of antigen; while in the last group, which served as a control, rabbit or guinea pig serum containing none of the specific antibody was introduced intravenously prior to injection of antigen. All intravenous injections were made by way of the jugular vein.

The antigens used for producing the antisera in rabbits were: (a) crystalline egg albumin; (b) horse serum; and (c) heat-killed virulent Friedländer's bacillus Type B. The same antigens were used for demonstrating hypersensitiveness in passively sensitized guinea pigs, with the exception that the type-specific polysaccharide of Friedländer's bacillus prepared according to the method of Heidelberger, Goebel, and Avery (6) was used to induce shock, instead of bacteria themselves.

The antisera used were: (a) pooled anti-crystalline egg albumin rabbit serum with a precipitin titer of 1:700,000; (b) pooled anti-horse rabbit serum with a precipitin titer of 1:10,000–1:20,000; (c) pooled anti-Friedländer rabbit serum which agglutinated Friedländer's bacillus in a dilution of 1:20–1:40. The undiluted antiserum precipitated the specific carbohydrate diluted 1:1,000,000.

Effect of Excess of Antibody on Hypersensitiveness to Egg Albumin

In order to determine whether protection against anaphylaxis is readily demonstrable when excess of antibody is present in the circulation, preliminary experiments were undertaken using the egg albumin anti-egg albumin system. In experiments made for orientation, such protection was not easily demonstrated. On the contrary, as may be seen from Table I, animals having an excess of circulating antibody appeared to exhibit an enhanced sensitivity as shown by the fact that they succumbed with symptoms of typical anaphylaxis upon

injection of less than 1 M.L.D. of antigen. These results suggest that excess of circulating antibody, far from protecting against the shock, may actually increase the susceptibility of the animals.¹

Unfortunately both the antialbumin and anti-horse sera were found to be toxic when introduced intravenously into the guinea pigs, so

TABLE I

The Effect of Excess of Circulating Antibody upon the Anaphylactic Response of Guinea Pigs Passively Sensitized with Anti-Egg Albumin Rabbit Serum

Guinea pig	Weight	Anti-egg rabbit serum (i.p.)	Interval	Additional anti-egg rabbit serum (i.v.)	Rabbit serum (anti-horse) (control) (i.v.)	Interval	Egg albumin antigen (i. v.)	No. of M.L.D. of antigen	Symptoms	Result
	gm.	cc.	hrs.	cc.	cc.	min.	cc.			
8-35	190	0.5	24				0.1	1	++++*	D*
8-42	175	"	"				0.1	1	++++	D
8-40	190	"	"				0.05	1/2	+++	S
8-45	205	"	"	1.5		55	0.05	1/2	++++	D
8-44	205	"	"	1.5		20	0.025	1/4	++++	D
8-43	212	"	"	1.5		20	0.0125	1/8	++++	D
8-48	180	"	"	1.5		20	0.00625	1/16	—	S
6-54	195	"	"		1.0	20	0.05	1/2	++++	D
4-00	187	"	"		1.0	20	0.025	1/4	—	S

* In this as well as in all subsequent tables, the symbols have the following meanings:

++++, intense symptoms; immediate death.

+++ , intense symptoms; survived.

++ , moderate but definite symptoms.

+ , very mild symptoms.

— , no symptoms.

S, survival.

D, anaphylactic death.

that all of the animals died an hour or more after intravenous injection of these antisera. While these toxic reactions could not be confused with the immediate and typical reactions characteristic of anaphylaxis they were, nevertheless, a disturbing element in the interpretation of

¹ It must be noted, however, that one of two controls tested with less than 1 M.L.D. of antigen also died.

TABLE II

The Effect of Excess of Circulating Antibody upon the Anaphylactic Response of Guinea Pigs Passively Sensitized with Anti-Friedländer Type B Rabbit Serum 1

Guinea pig	Weight	Anti-Friedländer Type B rabbit serum (i.p.)	Interval	Additional anti-Friedländer Type B rabbit serum (i.v.)	Rabbit serum (anti-horse) (control) (i.v.)	Interval	Friedländer Type B specific carbohydrate (i.v.)	No. of x.r.p. of carbohydrate	Symptoms	Result
	gm.	cc.	hrs.	cc.	cc.	min.	cc.			
7-97	217	0.5	24			20			++	S
8-93	235	"	"			"	0.4 1:10,000	1	++++	D
8-69	215	"	"			"			++++	D
8-81	212	"	"			"			-	S
7-96	215	"	"			"			+++	S
7-91	224	"	"			"	0.2 1:10,000	1/2	-	S
8-91	240	"	"			"			++++	D
8-90	252	"	"			"	0.1 1:10,000	1/4	+++	S
8-94	222	"	"			"			++	S
3-33	232	"	"	2		"	0.2 1:1000	5	++++	D
7-85	216	"	"			"			-	S
3-32	215	"	"	2		"	0.8 1:10,000	2	++++	D
3-42	212	"	"			"			++++	D
7-83	217	"	"			"			-	S
3-36	217	"	"	2		"	0.4 1:10,000	1	++	S
7-11	251	"	"			"			++++	D
7-03	232	"	"			"			-	S
7-50	243	"	"	2		"	0.2 1:10,000	1/2	++	S
7-13	235	"	"			"			+	S
7-08	242*	"	"	2		"	0.1 1:10,000	1/4	-	S
8-92	240*	"	"			"			+	S
7-18	229	"	"	2		"	0.2 1:40,000	1/8	+	S
7-07	231	"	"	2		"	0.1 1:40,000	1/16	-	S

* Two different lots of Friedländer antiserum used for intravenous injection, one in each of these two animals.

TABLE II—*Concluded*

Guinea pig	Weight	Anti-Friedländer Type B rabbit ser- um (i.p.)	Interval	Additional anti-Fried- länder Type B rab- bit serum (i.v.)	Rabbit serum (anti- horse) (control) (i.v.)	Interval	Friedländer Type B specific carbohydrate (i.v.)	No. of M.L.D. of carbo- hydrate	Symptoms	Result
3-20	232	0.5	24		2	20	0.4 1:1000	10	++++	D
3-35	241	"	"		2	"	0.2 1:1000	5	— ++++	S D
3-38	228	"	"			"				
7-84	216	"	"		2	"	0.8 1:10,000	2	— ++++	S D
3-49	215†	"	"			"			++++	D
3-52	227†	"	"			"			++++	D
7-86	222	"	"			"			—	S
3-48	233†	"	"		2	"	0.4 1:10,000	1	++++	D
3-47	232†	"	"			"			++++	D
7-61	250	"	"			"			+	S
7-64	250	"	"		2	"	0.2 1:10,000	1/2	—	S
7-49	237	"	"			"			++++	D
7-46	241	"	"		2	"	0.1 1:10,000	1/4	—	S

† Different lot of anti-horse rabbit serum used for intravenous injection of these four animals.

results. Because of this serum toxicity, too, the number of animals surviving for the final test was so small that the only conclusion drawn was that excess of circulating antibody does not appear to exert a protective effect.

Effect of Excess of Antibody on Hypersensitiveness to Friedländer's Bacillus Type B

With the hope of eliminating the difficulties attendant upon the use of toxic antisera, another series of experiments was undertaken in which anti-Friedländer Type B rabbit serum and the corresponding haptene were employed. This anti-serum proved to be nontoxic when injected intravenously. As may be seen from Table II, 0.4 cc. of a 1:10,000 dilution of carbohydrate appears to represent 1 M.L.D. of antigen for guinea pigs passively sensitized with 0.5 cc. of this anti-serum. From the table it appears that a slight degree of protection may be af-

fording occasionally merely by preliminary introduction of foreign serum (containing none of the specific antibody) as is shown by the fact that one sensitized guinea pig injected with anti-horse serum as control 20 minutes before inoculation of the specific carbohydrate survived 5 M.L.D. of antigen. It is also evident from the table that additional specific antibody introduced intravenously into similarly sensitized animals 20 minutes before the antigen has no very appreciable effect either in enhancing sensitivity or in affording any significant protection.

While it seemed evident from this and the preceding experiment that excess of antibody in the circulation affords no protection against anaphylaxis, it still remained undetermined whether circulating antibody enhances sensitivity as suggested by the first experiment (Table I). That such an increase in sensitivity actually does occur became clear from the experiments which follow.

Effect of Excess of Antibody on Hypersensitiveness to Horse Serum

In this set of experiments, the horse-anti-horse system was used, two lots of rabbit anti-horse serum being available. The first antiserum tested proved to be toxic when injected intravenously, but sufficient data were obtained from experiments with this antiserum to illustrate several important points concerning the effect of an excess of antibody on passive hypersensitiveness to horse serum. These results were controlled by intravenous inoculation of some of the passively sensitized animals with normal guinea pig, normal rabbit, or antialbumin instead of anti-horse rabbit serum previous to injection of the antigen (horse serum) (Table III).

From Table III it appears that 0.1 cc. of horse serum represents 1 M.L.D. of antigen for these passively sensitized animals. It is clear that here, as in the preceding experiment (Table II), foreign serum introduced parenterally into a sensitized animal tends to reduce its reactivity. Apparently, this reduction in reactivity is roughly related to the amount of foreign serum introduced, since large amounts of serum protect against greater quantities of antigen. It is interesting that even homologous normal serum (guinea pig) shows a protective effect which, however, is rather slight, in comparison with the protection afforded by rabbit serum. Kellaway and Cowell (7) have made a similar observation and have shown that the loss and return of reactivity in sensitized guinea pigs treated with normal guinea pig

TABLE III

The Effect of Excess of Circulating Antibody upon the Anaphylactic Response of Guinea Pigs Passively Sensitized with Anti-Horse Rabbit Serum 1

Guinea pig	Weight	Anti-horse rabbit serum (i.p.)	Interval	Additional anti-horse rabbit serum (i.v.)	Serum (control) (i.v.)			Interval	Horse serum antigen (i.v.)	No. of M.L.D. of antigen	Symptoms	Result
					Pooled, normal guinea pig	Pooled normal rabbit	Anti-albumin rabbit					
	gm.	cc.	hrs.	cc.	cc.	cc.	cc.	min.	cc.			
6-59	217	0.5	24						0.2	2	++++	D
6-64	233	"	"						0.1	1	++++	D
6-65	190	"	"						0.05	1/2	—	S
6-66	212	"	"								—	S
8-11	185	"	"	2				60	0.2	2	++++	D
8-19	185	"	"	1-2				60			++++	D
8-26	195	"	"	1-1.5				60	0.05	1/2	++++	D
8-32	175	"	"	1				60			++++	D
8-51	220	"	"	1				20	0.025	1/4	++++	D
6-88	220	"	"	1				20	0.0125	1/8	—	S
8-39	187	"	"		5			60	0.4	4	++++	D
8-57	207	"	"		5			60	0.2	2	—	S
8-33	215	"	"		3			60	0.1	1	++	S
8-41	195	"	"		2			60	0.1	1	++++	D
6-61	207	"	"			5		60	0.8	8	+++	S
6-62	215	"	"			4		60	0.6	6	+++	S
8-16	200	"	"			2		60	0.4	4	++++	D
8-18	185	"	"			2		60	0.2	2	—	S
8-20	200	"	"			1		60	0.2	2	++++	D
8-37	200	"	"			1		60	0.1	1	—	S
8-47	225	"	"				1.5	20	0.05	1/2	—	S
8-49	210	"	"				1.5	20	0.05	1/2	+++	S

serum may be correlated with a similar loss and return of reactivity of the smooth muscle.

In contrast with these control experiments, it will be seen that sensi-

TABLE IV

The Effect of Excess of Circulating Antibody upon the Anaphylactic Response of Guinea Pigs Passively Sensitized with Anti-Horse Serum 3

Guinea pig	Weight	Anti-horse rabbit serum (i.p.)	Interval	Additional anti-horse rabbit serum (i.v.)	Rabbit serum (control) (i.v.)		Interval	Horse serum antigen (i.v.)	No. of M.L.D. of antigen	Symptoms	Result
					Anti-Friedländer Type B	Anti-egg					
	gm.	cc.	hrs.	cc.	cc.	cc.	min.	cc.			
8-70	225	0.5	24				20	0.5	5	++++	D
3-27	252	"	"				"			—	S
8-73	220	"	"				"	0.1	1	++++	D
3-05	227	"	"				"			++++	D*
8-87	201	"	"				"			—	S
8-71	225	"	"				"	0.05	1/2	—	S
3-02	194	"	"				"			++++	D
3-23	237†	"	"				"			—	S
3-24	225†	"	"	2			"	0.2	2	++++	D
3-22	220†	"	"				"			++++	D
3-03	245	"	"				"			++	S
7-89	222	"	"	2			"	0.1	1	++++	D
7-11	224	"	"				"			++++	D
3-21	203†	"	"				"			++++	D
3-28	222†	"	"	2			"	0.05	1/2	++++	D
3-30	225†	"	"				"			++++	D
7-82	200	"	"				"			+++	S
7-63	250	"	"				"			+++	S
7-75	226	"	"	2			"	0.025	1/4	—	S
7-80	216	"	"				"			—	S
8-74	208	"	"	1			"			++++	D
8-72	206	"	"	1			"			+++	S
3-29	230†	"	"				"			—	S
8-84	227	"	"	2			"	0.0125	1/8	+++	S
8-83	212	"	"				"			++++	D
8-79	212	"	"				"			++++	D

* Death after ½ hour.

† Different lot of anti-horse rabbit serum used for intravenous injection in these animals.

TABLE IV—*Concluded*

Guinea pig	Weight	Anti-horse rabbit serum (i.p.)	Interval	Additional anti-horse rabbit serum (i.v.)	Rabbit serum (control) (i.v.)		Interval	Horse serum antigen (i.v.)	No. of M.L.D. of antigen	Symptoms	Result
					Anti-Friedländer Type B	Anti-egg					
8-77	gm. 212	cc. 0.5	hrs. 24	cc. 2	cc.	cc.	min. 20	cc. 0.00625	1/16	—	S
7-60	212	"	"				"				
7-87	212	"	"	2			"	0.8	8	++++	D
7-19	255	"	"				"			++++	D
7-65	222	"	"				"				
3-26	200	"	"	2			"	0.4	4	—	S
7-01	233	"	"				"			++++	D
7-70	216	"	"				"			++++	D
7-69	185	"	"	2			"	0.2	2	—	S
3-37	208	"	"				"			++	S
3-23	208	"	"	2			"			++++	D
3-39	206	"	"				"	0.1	1	—	S
7-98	224	"	"				"			++++	D
8-88	204	"	"		2		"	0.1	1	+	S
8-85	221	"	"				"			+	S
8-78	212	"	"		2		"			++++	D
8-75	187	"	"		1		"	0.05	1/2	+	S
7-99	230	"	"				"	0.05	1/2	+++	S
3-01	209	"	"				"			++++	D
3-04	207	"	"				"				
3-07	232	"	"	2			"	0.025	1/4	—	S
3-40	232	"	"				"			—	S
3-25	207	"	"	2			"	0.0125	1/8	+	S
										—	S

tized animals which had received additional specific antibody previous to introduction of antigen, failed to tolerate even 2 M.L.D. of antigen. Furthermore, two animals died when injected with 0.05 cc. and one when injected with 0.025 cc. of horse serum, that is, 1/2 and 1/4 M.L.D.

respectively. The important point here, then, is that rabbit and even guinea pig serum containing no specific antibody, when introduced intravenously into sensitized guinea pigs before testing with antigen, reduces reactivity while the introduction of rabbit serum containing the specific antibody does not reduce but rather tends to enhance sensitivity. These results are in agreement with those reported earlier with antialbumin serum (Table I).

Further corroboration was obtained with a second anti-horse rabbit serum which was not toxic for guinea pigs. The results were controlled by determining the effect of intravenous inoculation of anti-Friedländer or anti-egg rabbit serum upon the hypersensitive response of guinea pigs passively sensitized with 0.5 cc. of this anti-horse serum. The interval between injection of these antisera and the horse serum antigen was 20 minutes (Table IV).

Here again, it may be seen that the mere introduction of foreign serum may reduce slightly the reactivity of the sensitized animal. Hence, the protection afforded by antibody-containing serum (if it occurs at all) should be attributed to its action as a foreign protein rather than to its antibody-content. It is more significant, however, that several of the animals given an excess of antibody-containing serum died when tested with less than 1 M.L.D. of antigen. Thus, three died after introduction of $1/2$ M.L.D., one after $1/4$ M.L.D., and two after $1/8$ M.L.D., while only one animal out of nine died when tested with less than 1 M.L.D. of antigen after being given an excess of other than specific antiserum. The enhancement of sensitivity observed in the present experiments would indicate that similar, though less extensive, findings in previous experiments were not accidental. These findings are particularly significant because foreign serum containing none of the specific antibody tends to reduce the reactivity of sensitized animals when injected in comparable amounts. As a consequence, the actual increase in sensitivity conferred by an excess of antibody might have been even greater than that recorded here were it not for the fact that a nonspecific foreign protein reaction which tends to decrease the animal's reactivity was occurring at the same time.

The summary of the results of experiments in guinea pigs passively sensitized with both lots of anti-horse serum is presented in Table V.

This table indicates in a graphic manner the essential features of these experiments. Guinea pigs passively sensitized with anti-horse rabbit serum and injected with additional antibody show a definitely greater sensitivity than animals similarly sensitized, but given an additional inoculation of other rabbit sera containing none of the specific antibody. This increase in sensitivity is demonstrable in two ways: (a) Death occurs in some animals upon injection of amounts of antigen

TABLE V

Composite Results of Passive Sensitization of Guinea Pigs with 0.5 Cc. of Anti-Horse Rabbit Serum, Showing Effect of Subsequent Introduction of Excess of Antibody into the Circulation

		No. of M.L.D. of horse serum				
		2	1	1/2	1/4	1/8
Without injection of additional antiserum	No. of animals tested		4	5		
	No. of deaths		3	1		
	Percentage of deaths		75%	20%		
With injection of additional anti-horse rabbit serum previous to testing with antigen	No. of animals tested	5	3	5	6	6
	No. of deaths	4	2	5	2	2
	Percentage of deaths	80%	66%	100%	33%	33%
With injection of rabbit serum (control) previous to testing with antigen	No. of animals tested	5	6	5	4	2
	No. of deaths	2	2	1	0	0
	Percentage of deaths	40%	33%	20%	0%	0%

M.L.D. of antigen = 0.1 cc.

less than 1 M.L.D., and (b) when tested with 1 or 2 M.L.D. of antigen the mortality rate among animals receiving excess of antibody is considerably higher than among those which received other rabbit sera.

DISCUSSION

The preceding experiments were undertaken because of the prevalent view that the presence of an excess of antibody in the circulation is responsible for the refractoriness to anaphylaxis manifested by some hypersensitive animals. This concept is based largely on the work of Weil (1) quoted previously and on other experiments of his (8) in

which he showed that guinea pigs actively sensitized with large amounts of antigen became hypersensitive more slowly and required larger amounts of antigen for induction of fatal shock than guinea pigs sensitized with small amounts of antigen. This he attributed to the fact that the former animals had more circulating antibody than the latter. Even if this quantitative difference in the amount of antibody formed in these two groups of animals does occur, which in the light of immunological experience is rather doubtful, it is not necessarily the only explanation of the greater refractoriness of animals sensitized with large amounts of antigen, as will be shown later.

This concept, however, has led even such an authority as Wells (9) to state that "this term [antianaphylaxis] should logically be applied only to a resistance due to antibodies." From the experiments recorded in this paper, however, it would appear that an excess of antibody in the circulation does not establish a state of antianaphylaxis. On the other hand, some protection against anaphylactic shock may be induced nonspecifically merely by the introduction of serum whether containing the specific antibody or not. This is in agreement with numerous reports in the literature concerning the capacity of a wide variety of unrelated substances to render sensitized animals refractory to anaphylaxis (hypertonic salt, alkalis, mineral waters, saponin, lipoids, foreign sera, narcotics, hirudin, etc. (10, 11)). In the light of our results, then, it is extremely likely that the occasional instances of protection noted in our experiments in sensitized animals inoculated with additional antibody, as well as a similar though more extensive protection reported by Weil, are attributable to the nonspecific effect of the serum injected. It is also possible that traces of antigen remaining in the antiserum which was injected intravenously into the already hypersensitive animal may have caused some degree of specific desensitization. It may be pertinent to point out here that persistence of antigen in animals undergoing active sensitization is also a very likely explanation of Weil's failure to establish as high a degree of reactivity in animals sensitized with large amounts of antigen, as contrasted with those inoculated with smaller quantities. Antibody may be demonstrated in the circulation of animals undergoing active sensitization often before all of the antigen

has been eliminated, and hence ideal conditions for specific desensitization are present. It is natural that larger amounts of antigen persist longer than smaller quantities and this fact, not the greater amount of circulating antibody, may account for Weil's results. That the same fundamental mechanism may underly specifically or nonspecifically induced antianaphylaxis will be elaborated upon in a subsequent publication.

While the present experiments have shown that circulating antibody is not responsible for a state of antianaphylaxis they have indicated in addition, that frequently those animals which have received an excess of antibody react to smaller quantities of antigen than animals which have not received an injection of additional antibody. It should be emphasized that in these experiments a period of only 20 minutes was allowed to elapse between the intravenous injections of additional antiserum and of antigen into the hypersensitive animals (although in some instances the interval was 1 hour). In any event, the interval was shorter than that which has usually been acknowledged to be the minimum incubation period for the development of passive hypersensitiveness in the guinea pig, during which time it is claimed the antibody becomes fixed in the cell. These experiments, then, suggest the possibility that anaphylaxis is determined by both circulating and fixed antibody, and that circulating antibody, far from being a protective mechanism for the hypersensitive cell, actually increases the degree of sensitivity.

This view of the rôle of circulating antibody receives considerable support from the work of Kellett (12) who showed that it is possible to induce anaphylaxis in guinea pigs by injecting antiserum 45 minutes after the specific antigen. Indeed, the recent work of Zinsser and Enders (13) has shown that some guinea pigs may be thrown into fatal shock when an interval as little as $1\frac{1}{2}$ minutes intervenes between the injections of antigen and antiserum, and that this phenomenon may be demonstrated regardless of whether the antigen or antiserum is the first to be injected. It is admitted (14-16) that circulating antibody may be responsible in some part for the anaphylactic reactivity of dogs, mice, and rabbits, and hence not unlikely it should play a part in the reactivity of guinea pigs.

The failure of some of the sensitized animals in the present experiments to manifest an increased reactivity when given additional

antibody may possibly be attributed to individual variation in the animals, to the nonspecific effects induced by introduction of additional antiserum, or to traces of antigen remaining in the antiserum.

The experiments reported here tend to invalidate the idea that anaphylaxis is due to fixed antibody alone, and that refractoriness to anaphylaxis is due to circulating antibody. The precise mechanism of antianaphylaxis and its relation to anaphylaxis and to immunity (resistance to infection) will be discussed in a subsequent publication.

CONCLUSIONS

1. Sensitized guinea pigs injected with normal rabbit or guinea pig serum previous to intravenous inoculation of antigen may be protected against a few lethal doses of antigen. The protection is greater with foreign than with homologous serum and appears to be related roughly to the amount of serum introduced.

2. Sensitized guinea pigs injected with antibody-containing serum preliminary to intravenous injection of antigen, show no greater refractoriness to anaphylaxis than do those injected with normal serum.

3. Moreover, in many instances, the injection of an excess of antibody into the circulation of sensitized guinea pigs, leads to an increased susceptibility of these animals to anaphylaxis.

4. These results indicate that an excess of circulating antibody is not responsible for a state of antianaphylaxis, but on the contrary, may contribute toward the anaphylactic reaction itself.

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THE RELATION BETWEEN ANTIANAPHYLAXIS AND ANTIBODY BALANCE

II. THE EFFECT OF SPECIFIC DESENSITIZATION UPON RESISTANCE TO INFECTION AND UPON ANTIBODY BALANCE

BY MARION C. MORRIS, PH.D.

(From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis)

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Specific desensitization is currently explained on the basis of an hypothesis that the introduction of specific antigen into a sensitized animal causes a saturation or partial depletion of the cellular antibody responsible for hypersensitiveness. However, in view of the many reports in the literature (1, 2) and our own results (3) which indicate that refractoriness to anaphylaxis may be induced nonspecifically, it seemed important to determine whether the antibody balance is actually involved in specific desensitization. It appeared, too, that such a study should be of value in establishing a consistent concept of the relation between the states of anaphylaxis, antianaphylaxis, and immunity.

The Effect of Specific Desensitization upon Subsequent Resensitization

If specific desensitization be due to a saturation of cellular antibodies, it should be possible to resensitize previously desensitized animals merely by supplying additional antibody. Accordingly, a group of guinea pigs was sensitized, specifically desensitized, and later resensitized, and their response to the parenteral introduction of antigen was compared with that of normal animals passively sensitized with the same antiserum. The method of passive sensitization was used throughout in order to avoid the complications incident to individual differences in the capacity of the animals to produce antibodies by active sensitization.

A group of guinea pigs was passively sensitized by intraperitoneal injection of 0.5 cc. pooled anti-horse serum (titer 1:10,000). After 24 or 48 hours these guinea pigs were specifically desensitized by an intraperitoneal injection of horse serum as indicated in Table I. In some instances, a second intravenous injection of antigen was given at a later time to increase further the degree of desensitization. After a further interval of 24 to 48 hours these guinea pigs were again sensitized with 0.5 cc. of the same antiserum and after an incubation period of 24 hours were tested by injection of horse serum into the jugular vein.

TABLE I

The Effect of Desensitization upon Subsequent Passive Resensitization of Guinea Pigs (Horse Serum-Anti-Horse Serum System)

Guinea pig	Weight	Anti-horse rabbit serum for first sensitization (i.p.)	Interval	Horse serum antigen for desensitization	Interval	Anti-horse rabbit serum for resensitization (i.p.)	Interval	Horse serum antigen (i.v.)	No. of M.L.D. of antigen	Result
	gm.	cc.	hrs.	cc.	hrs.		hrs.	cc.		
8-85	221	0.5	24	0.05 (i.p.)	48	0.5	24	0.1	1	S
8-25	202	"	48	0.1 (i.p.)	24	"	"	0.2	2	S
8-84	227	"	24 72	0.025 (i.p.) 0.1 (i.v.)	24	"	"	0.4	4	S
8-86	190	"	24 72	0.075 (i.p.) 0.1 (i.v.)	24	"	"	0.6	6	S
Controls										
6-66	212	0.5	24					0.05	1/2	S
6-64	223	0.5	24					0.1	1	D
6-59	217	0.5	24					0.2	2	D

D, died with symptoms typical of anaphylaxis.

S, survived.

When the response of these resensitized antianaphylactic animals is compared with that of normal animals passively sensitized with the same amount of anti-horse serum, it is clear that the former animals have a greater tolerance for the specific antigen than the latter. This suggests that antibody depletion is not an adequate explanation of the effect of desensitization since, if this were so, it might be expected that

desensitized animals would have become at least as highly sensitized as normal animals upon receiving the same amount of antibody.

TABLE II

The Effect of Desensitization upon Subsequent Passive Resensitization of Guinea Pigs (Pneumococcus Type II Carbohydrate-Antipneumococcus Serum System)

Guinea pig	Weight		Interval	Pneumococcus Type II carbohydrate for desensitization		Interval	Antipneumococcus Type II rabbit serum for resensitization (i.p.)		Interval	Pneumococcus Type II carbohydrate (i.v.)		No. of m.l.n. of carbohydrate	Result
	gm.	cc.	hrs.	cc.		hrs.	cc.		hrs.	cc.			
7-52	231	0.5	24	0.1	1:1000 (i.v.)	2	0.5	24	0.1	1:100		5	S
7-46	235	"	24	0.1	1:1000 (i.v.)	2	"	"	0.3	1:100		15	D
1-14	200	"	8 29	0.3 1:1000 (i.p.) 0.05 1:200 (i.v.)		1/2	"	"	0.3	1:100		15	S
1-79	240	"	24 25	0.1 1:2000 (i.v.) 0.2 1:100 (i.v.)		48	"	"	0.3	1:100		15	S
1-80	215	"	24 25	0.1 1:2000 (i.v.) 0.8 1:100 (i.v.)		48	"	"	0.8	1:100		40	S
1-84	182	"	24 25	0.1 1:2000 (i.v.) 0.4 1:100 (i.v.)		48	"	"	0.8	1:100		40	S
1-81	185	"	24 25	0.1 1:2000 (i.v.) 0.8 1:100 (i.v.)		48	"	"	0.8	1:100		40	S
Controls													
7-45	235	0.5	24							0.1 1:1000		1/2	S
7-42	192	0.5	24							0.2 1:1000		1	D
7-44	223	0.5	24							0.2 1:1000		1	D

D, died with symptoms typical of anaphylaxis.

S, survived.

A similar experiment was carried out with pooled antipneumococcus Type II serum (titer 1:80-1:160) and the corresponding carbohydrate haptene (reactive by precipitation in a dilution of 1:100,000) (Table II).

The results of this experiment are in complete agreement with those of the preceding one. They show clearly that desensitization cannot be attributed to saturation of the antibody. While the exact extent of the desensitization was not determined, it is evident that the resensitized antianaphylactic animals withstood at least 40 times the

TABLE III

The Effect of Desensitization upon Subsequent Passive Resensitization of Guinea Pigs with an Unrelated Antiserum

Guinea pig	Weight	Rabbit antiserum for sensitization		Interval	Homologous specific carbohydrate for desensitization	Interval	Anti-horse rabbit serum for sensitization(i.p.)	Interval	Horse serum antigen (i.v.)	No. of M.L.D. of antigen	Result
		Anti-Friedländer Type B	Antipneumococcus Type II								
	gm.	cc.	cc.	hrs.	cc.	hrs.	cc.	hrs.	cc.		
8-92	229	2 (i.v.)		72	0.1 1:10,000 (i.p.)	5	0.5	24	0.2	2	S
7-08	252	2 (i.v.)		72	0.1 1:10,000 (i.p.)	"	"	"	0.4	4	S
7-15	231	0.5 (i.p.)		24	0.1 1:10,000 (i.v.)	"	"	"	0.8	8	S
				96	0.1 1:10,000 (i.p.)	"	"	"			
7-03	232	2 (i.v.)		48	0.1 1:10,000 (i.p.)	"	"	"	0.9	9	S
7-12	222		0.5 (i.p.)	24	0.1 1:1000 (i.p.)	"	"	"	2	20	S
7-09	202		0.5 (i.p.)	24	0.1 1:1000 (i.p.)	"	"	"	2	20	S
Controls											
6-66	212						0.5		0.05	1/2	S
6-64	233						0.5	24	0.1	1	D
6-59	217						0.5		0.2	2	D

D, died with symptoms typical of anaphylaxis.
S, survived.

amount of antigen which killed normal guinea pigs passively sensitized with the same amount of antiserum.

The preceding experiments, however, seemed open to the possible objection that resensitization might have been ineffective because the antigen used for desensitization might still have been present at the

tissue surfaces and might have combined with a fresh supply of the same antibody, thus preventing it from reaching the cells.

To see whether this might be true, guinea pigs which had been passively sensitized with pooled anti-Friedländer Type B or antipneumococcus Type II serum were specifically desensitized with the respective haptenes. After a period of 5 hours, all of the guinea pigs were again passively sensitized, but this time with anti-horse serum (Table III).

The results of this experiment indicate that animals which have been specifically desensitized by Friedländer or pneumococcus carbohydrates, fail to react when resensitized with anti-horse serum and tested with the corresponding antigen. These results are in complete accord with those obtained previously and seem to supply conclusive evidence that desensitization is not due to a blocking of cellular antibody. This type of experiment is particularly instructive because the evidence is valid regardless of whether a saturation of cellular or humoral antibodies is postulated as the essential prerequisite for antianaphylaxis.

The Effect of Specific Desensitization upon Resistance to Infection

Preceding experiments have shown that the refractory state following desensitization does not depend upon antibody depletion, since anaphylactic reactivity is not restored by supplying additional antibody. It is conceivable, however, that concurrently with any changes caused by the injection of antigen into hypersensitive animals and directly responsible for a state of antianaphylaxis, some antigen may combine with and neutralize some portion of the antibody. If such be the case, and if an appreciable amount of antibody be thus depleted by the process of desensitization there might occur, as a consequence, a change in the specific resistance to infection of such animals. This possibility was investigated by comparing the relative resistance to infection with Friedländer's bacillus Type B of two series of guinea pigs: (a) animals passively sensitized with anti-Friedländer serum; and (b) animals similarly sensitized but, in addition, desensitized with the specific hapten.

Having determined the lethal amount of the specific carbohydrate (reactive by precipitation in a dilution of 1:1,000,000) for guinea pigs passively sensitized

with 1 cc. of pooled anti-Friedländer Type B rabbit serum (titer 1:20-1:40) (Table IV) another series of guinea pigs was similarly passively sensitized and 8 hours later desensitized with a sublethal amount of the polysaccharide injected intraperitoneally. The interval elapsing between the time of sensitization and final test with carbohydrate injected into the jugular vein was the same in all animals (28 to 29 hours). It will be seen that the method of desensitization used was effective

TABLE IV

Minimum Lethal Dose of Antigen (Specific Carbohydrate) for Guinea Pigs Passively Sensitized with Anti-Friedländer Type B Rabbit Serum (Lot 3)

Guinea pig	Weight	Anti-Friedländer rabbit serum (i.p.)	Interval	Friedländer Type B specific carbohydrate 1:10,000 dilution (i.v.)	Symptoms	Result
	gm.	cc.	hrs.	cc.		
3-53	211	1.0	28-29		—	S
3-60	187	"	"	0.1	+	S
3-55	232	"	"		—	S
3-61	198	"	"	0.2	++++	D
3-68	232	"	"		—	S
3-70	232	"	"	0.4	—	S
3-63	195	"	"		++++	D
3-66	245	"	"		—	S
3-72	222	"	"	0.8	++++	D
2-21	195	"	"		++++	D
2-22	205	"	"		++++	D

M.L.D. = 0.8 cc. 1:10,000 dilution.

++++, intense symptoms; immediate death.

+++ , intense symptoms; survived.

++ , moderate but definite symptoms.

+ , very mild symptoms.

—, no symptoms.

S, survived.

D, anaphylactic death.

to the extent that desensitized animals survived at least 60 M.L.D. of the specific carbohydrate (Table V). Unfortunately it was not possible to test the animals with larger quantities of carbohydrate because of the limited supply of this material available, so that the exact lethal dose for the desensitized guinea pigs could not be definitely established.

Having secured a degree of desensitization permitting the animals to withstand at least 60 times the ordinary lethal dose of antigen an attempt was made to

determine the effect of such desensitization upon resistance of these animals to infection. Table VI shows the results obtained in experiments in which a series of guinea pigs comprising both sensitized and desensitized animals was tested for resistance to infection following intraperitoneal injection of varying amounts of virulent culture of Friedländer's bacillus Type B. All of the guinea pigs were observed for 4 days and Friedländer's bacillus was isolated from the blood of animals which died.

TABLE V

Result of Desensitization with the Specific Carbohydrate of Friedländer's Bacillus Type B in Guinea Pigs Passively Sensitized with Anti-Friedländer Type B Rabbit Serum (Lot 3)

Guinea pig	Weight	Anti-Friedländer Type B rabbit serum (i.p.)	Interval	Friedländer Type B carbohydrate (i.p.) (1:10,000 dilution)	Interval	Friedländer Type B carbohydrate (i.v.) (1:1000 dilution)	No. of M.L.D. of carbohydrate	Symptoms	Result
	gm.	cc.	hrs.	cc.	hrs.	cc.			
3-65	235	1	8	0.2	20-21	0.8	10	+	S
3-64	204	"	"	"	"	"	20	-	S
1-01	205	"	"	"	"	1.6		+++	S
3-67	207	"	"	"	"	"	30	-	S
2-29	201	"	"	"	"	2.4		+++	S
2-20	210	"	"	"	"	4.8	60	+	S

+++ , intense symptoms; survived.

+, very mild symptoms.

-, no symptoms.

As will be seen from the results presented in Table VI no appreciable differences in resistance were detected, although anaphylactic reactivity in the desensitized animals had been decreased so that they withstood at least 60 M.L.D. of haptene (Table V). These results support the earlier conclusion that desensitization is not due to antibody depletion.

The Effect of Specific Desensitization upon the Content of Circulating Antibody

While the data presented above appear to indicate that desensitization does not deplete the antibody, it seemed essential to make a more

direct comparison of the antibody content of the serum of sensitized and of desensitized guinea pigs. Because of the fact that the antibody which was introduced to effect passive sensitization of the guinea pigs was highly diluted by the total volume of the blood of the animal, precipitation and complement fixation were found to be inadequate tests for its detection. An attempt was made, therefore, to determine whether a mouse protection test might serve for this purpose.

TABLE VI

The Effect of Desensitization upon Resistance to Infection of Guinea Pigs Passively Sensitized with Anti-Friedländer Type B Rabbit Serum (Lot 3)

	Virulent Friedländer's bacillus Type B (i.p.)					
	10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.	10 ⁻⁴ cc.	10 ⁻⁵ cc.	10 ⁻⁶ cc.
A						
Guinea pigs passively sensitized intra-peritoneally with 1 cc. anti-Friedländer Type B rabbit serum. Tested 28-29 hrs. after sensitization	2 D 1 S	2 D 6 S	1 D 1 S	3 S		
B						
Guinea pigs passively sensitized as in A. Desensitized 8 hrs. after sensitization by intraperitoneal injection of 0.2 cc. 1:10,000 dilution of the specific carbohydrate. Tested 28-29 hrs. after sensitization	1 D 2 S	3 D 5 S	1 D 2 S	2 S		
C						
Normal guinea pigs					2 D 1 S	3 D 1 S

D, died.
S, survived. } Under observation for 4 days.

In a preliminary experiment, therefore, a series of mice was injected with 0.2 cc. of mixtures consisting of equal volumes of serial dilutions of anti-Friedländer Type B serum and of standard dilutions of a broth culture of Type B Friedländer's bacillus (18 hours old). That the number of organisms injected each time was the same in all mixtures of the same dilution was ascertained by plating serial dilutions of the culture used in each experiment. The mice were observed for 4 days and Friedländer's bacillus was isolated from the heart's blood of all animals

succumbing to infection. Results of this experiment which comprised over 150 mice are presented in Table VII, in terms of percentage of animals surviving infection.

The results of this preliminary experiment indicated that the mouse protection method was delicate enough for the purpose at hand, since fair protection of mice was still evident when the antiserum was diluted 1:800. Therefore, fifteen guinea pigs were passively sensitized by intraperitoneal injection of 1 cc. of anti-Friedländer Type B serum and divided into two groups. The first group (Table VIII) comprising eight animals was bled from the heart 28 to 29 hours after sensitization. The second group (Table IX) comprising seven animals was desensitized 8 hours after sensitization by intraperitoneal injection of 0.2 cc. of 1:10,000 dilution of the specific polysaccharide, and bled from the heart 20 to 21 hours later. The antibody content of the serum of these two groups of guinea pigs was determined by injecting a number of mice with 0.2 cc. of a mixture of equal volumes of the

TABLE VII

Protection of Mice with Varying Dilutions of Anti-Friedländer Type B Serum (Lot 3)

0.1 cc. of Friedländer's bacillus Type B diluted	0.1 cc. of anti-Friedländer Type B serum (lot 3) diluted					Controls without antiserum
	1:50	1:100	1:200	1:400	1:800	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1:1000	75	60	30	0		0
1:10,000	71	63	72	52	10	0
1:100,000						0
1:1,000,000						25

Figures represent percentage of survivals (4 days).

serum of these guinea pigs diluted as indicated in the tables and a 1:10,000 dilution of an 18 hour broth culture of Friedländer's bacillus (approximately 100 M.L.D.). Here, too, the number of organisms injected was always determined by plating serial dilutions of the culture. The mice were observed for 4 days, at the end of which time the respective number of deaths and survivals was recorded (Tables VIII and IX). Friedländer's bacillus was always isolated from the heart's blood of mice which died.

Upon comparison of results of mouse protection by sera of sensitized and of desensitized guinea pigs respectively, it appears that the serum of sensitized animals has a somewhat greater protective capacity than that of desensitized animals. When the results are judged according to the previous standardization of the same antiserum (Table VII), it would appear that the protection afforded by a 1:5 dilution of the

sera of sensitized guinea pigs (see Table VIII) corresponds to a 1:200 dilution of the original anti-Friedländer serum, while that afforded by a 1:10 dilution of the sensitized guinea pig sera corresponds to a 1:400 dilution of the original antibody. On the other hand, the protection afforded by a 1:5 dilution of sera of desensitized

TABLE VIII

Protection Tests in Mice with the Serum of Guinea Pigs Sensitized with Anti-Friedländer Type B Rabbit Serum (Lot 3)

Guinea pig	Weight	Mice inoculated with 0.1 cc. Type B Friedländer's bacillus dilution 1:10,000 and 0.1 cc. guinea pig* serum diluted	
		1:5	1:10
	gm.		
3-44	205	4 S	4 D 1 S
3-59	190	5 S	1 D 4 S
3-62	190	3 D 2 S	4 D 1 S
3-69	220	5 D	5 D
3-71	208	1 D 4 S	1 D 4 S
Pooled			
2-60	216		
2-62	177	1 D 9 S	3 D 7 S
2-63	194		
Total No. mice tested.....		34	35
Total No. survivors.....		24	17
Percentage survivors.....		70%	49%

D, died within 4 days.

S, survived 4 days.

* Guinea pigs sensitized with 1 cc. anti-Friedländer serum, bled 28 to 29 hours later.

guinea pigs (see Table IX) represents a little more than the equivalent of a 1:400 dilution of the original antiserum, while that afforded by a 1:10 dilution of desensitized guinea pig sera represents somewhat more than a 1:800 dilution of the original antibody. These figures indicate the possibility that desensitization has decreased the antibody content of the serum by one-half. These differences in protection of

mice, however, are far too slight to account for the high degree of refractoriness to anaphylaxis manifested by the desensitized guinea pigs. Moreover, in view of the experiments in which both the sensitized and desensitized guinea pigs were found to be equally resistant to infection (Table VI), it seems probable that the differences in

TABLE IX

Protection Tests in Mice with the Serum of Guinea Pigs Sensitized with Anti-Friedländer Type B Rabbit Serum (Lot 3) and Desensitized with the Specific Carbohydrate

Guinea pig	Weight	Mice inoculated with 0.1 cc. Type B Friedländer's bacillus dilution 1:10,000 and 0.1 cc. guinea pig* serum diluted	
		1:5	1:10
	gm.		
2-55	205	1 D 3 S	4 D 1 S
2-56	237	2 D 3 S	3 D 2 S
2-57	235	2 D 3 S	3 D 2 S
2-58	262	4 D 1 S	5 D
Pooled			
2-59	187		
2-61	200	3 D 6 S	9 D 1 S
2-64	175		
Total No. mice tested.....		28	30
Total No. survivors.....		16	6
Percentage survivors.		57%	20%

D, died within 4 days.

S, survived 4 days.

*Guinea pigs sensitized with 1 cc. anti-Friedländer serum, specifically desensitized after a period of 8 hours and bled 20 to 21 hours later (28 to 29 hours after original sensitization).

antibody concentration as shown by mouse protection tests might be due in part at least to inadequacy of the method of testing. In the light of experiments of Sia (4) who showed that soluble specific substance enhances the growth of *Pneumococcus in vitro*, there is the possibility that the presence of small amounts of carbohydrate in the serum of the desensitized animals and not the reduction in its anti-

body content may be responsible for the higher mortality of mice in the protection tests with the sera of desensitized guinea pigs.

DISCUSSION

A number of investigators who have maintained that specific desensitization exhausts or neutralizes the antibody responsible for hypersensitiveness have been confronted with facts incompatible with this hypothesis. Thus, Weil and Coca (5) found that passively sensitized guinea pigs, after desensitization, required a larger amount of antialbumin serum for resensitization than was necessary for the primary sensitization. A control group of guinea pigs inoculated with normal rabbit instead of antialbumin rabbit serum and later with egg albumin as used for desensitization in the test group, also required larger amounts of antiserum for sensitization than normal animals. Because of the behavior of this control group, Weil and Coca concluded that antianaphylaxis was actually due to a neutralization of antibody. Their interpretation of these results seems open to question. The mere fact that in both groups of animals it was more difficult to elicit the anaphylactic response than in normal passively sensitized animals indicates the non-specific nature of the state of antianaphylaxis. Furthermore, other investigators (6-11) have shown that animals sensitized with two antigens when desensitized with one become refractory to the other also. Friedberger and his collaborators (8) claimed that refractoriness to the antigen used for desensitization was so much greater than refractoriness to the other antigen that antianaphylaxis must be of a dual nature consisting of (a) a general refractoriness to all anaphylatoxin, and (b) a true specific antianaphylaxis. Bessau (6, 7), however, was unable to show any such quantitative differences and claimed that the state of antianaphylaxis was entirely nonspecific. It should be emphasized that none of these investigators made any direct determination of the antibody content of these animals to show that a neutralization of antibody takes place.

In experiments presented in this paper, it has been shown that specifically desensitized guinea pigs when resensitized with the same or with an unrelated antiserum fail to attain the same degree of hypersensitive reactivity as normal animals sensitized with the same amount of these antisera. These facts would appear incompatible with any theory which maintains that a neutralization of either fixed or circulating antibody is the principal cause of antianaphylaxis. Experiments of this kind, however, while offering evidence that antianaphylaxis is due to nonspecific changes in the animal body, do not supply direct evidence concerning the effect of specific desensitization upon the content of cellular antibody, since, as a matter of fact, no methods exist by which such a quantitative determination may be made. Indirect determination of the effect of specific desensitization

upon the content of cellular antibody may be made, however. The methods used in the present experiments were founded upon the well known fact that hypersensitiveness to the carbohydrate of Friedländer's bacillus and resistance to infection in sensitized animals are both due to the same type-specific antibody directed against the capsular polysaccharide. Hence, if desensitization by the specific carbohydrate depletes the antibody so that hypersensitivity is decreased, a corresponding decrease in the animal's resistance to infection should occur. However, it was found that hypersensitiveness may be reduced by desensitization at least 60 times without affecting the resistance of the animal to infection. It is logical to conclude, therefore, that specific desensitization does not affect appreciably the quantity of fixed or circulating antibody present in the sensitized animal. Furthermore, a direct study of the antibody content of the serum of guinea pigs before and after specific desensitization has shown that a decrease in antibody content commensurate with a 60-fold decrease in hypersensitiveness does not occur. If a considerable decrease in the content of cellular antibody had occurred, it is likely that more antibody would have been taken up by the cells from the circulation, and hence a decrease in cellular antibody should have been followed by a significant decrease in serum antibody. Such was not found to be the case.

These experiments taken in conjunction with those of the previous publication (3) lead to the conclusion that refractoriness to anaphylaxis is not dependent upon an excess of circulating antibody nor upon antibody depletion, but is occasioned by nonspecific changes in the animal.

Thus, for instance, Eggstein (12), showed that the alkaline reserve of the plasma is lowered during shock and that administration of sodium carbonate to sensitized dogs or guinea pigs has a protective effect if given before the shocking injection of antigen. Kopaczewski (13) claimed that those substances which lower the surface tension or increase the viscosity of the blood are effective anti-anaphylactic agents. Widal, Abrami, and Vallery-Radot (14) believed that the state of antianaphylaxis is brought about by a change in the physical equilibrium of the animal. Most adequate as a plausible explanation of the mechanism of antianaphylaxis, however, is the hypothesis of Bronfenbrenner (15-17) who has shown that following anaphylactic shock the capacity of the blood of the surviving animals to inhibit tryptic activity is increased. It is this antitryptic property which prevents the activity of digestive ferments necessary for the formation

body content may be responsible for the higher mortality of mice in the protection tests with the sera of desensitized guinea pigs.

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of the poisonous split products responsible for anaphylaxis. While such changes responsible for a temporary refractoriness to anaphylaxis are induced readily by the specific antigen-antibody combination, Bronfenbrenner showed that many nonspecific agents which raise the antitryptic index of the blood also produce the same resistance to anaphylaxis. Hence, the fundamental mechanism underlying specifically or nonspecifically induced refractoriness to anaphylaxis is essentially the same.

If this be the relation between anaphylaxis and resistance to anaphylaxis it is obviously quite different from the relation existing between anaphylaxis and resistance to infection. Anaphylaxis, as indicated in the previous publication (3) is in all probability, due to the events which follow upon the combination of antigen with both fixed and circulating antibody. Resistance to infection is dependent upon the identical antibodies, both cellular and humoral. The essential difference between anaphylaxis and immunity to infection depends upon the manner of testing the animal which has such antibodies. If the specific antigen be given quickly, in sufficient quantity, in an easily utilizable form to the sensitized animal (such as happens when the specific carbohydrate is inoculated intravenously into guinea pigs sensitized with anti-Friedländer Type B serum) toxic products are liberated and the manifestation of reaction is one of acute shock which is called anaphylaxis. If, on the other hand, the antigen be given in smaller quantities and in a less utilizable manner (such as occurs when infection with living bacteria takes place), this immediate reaction is not detectable in the gross. In this latter case there is sufficient time for the defense mechanisms of the body to become operative (phagocytosis, lysis, etc.) and the manifestation is one of resistance to infection which is called immunity.

The state of antianaphylaxis, on the other hand, is due to nonspecific changes in the animal organism which inhibit rapid production of toxic products directly responsible for anaphylactic shock. It may be induced specifically or nonspecifically but in any case is not concerned with the relative distribution of cellular and humoral antibodies.

CONCLUSIONS

It has been shown that antianaphylaxis is not caused by a partial saturation of cellular or humoral antibodies by the following facts.

1. Guinea pigs passively sensitized with anti-horse or antipneumo-

coccus serum and specifically desensitized do not manifest as great a reactivity upon resensitization with the same antiserum as upon the original sensitization.

2. Guinea pigs passively sensitized with anti-Friedländer Type B serum or antipneumococcus Type II serum and specifically desensitized do not attain the same degree of reactivity as normal animals when passively sensitized with anti-horse serum.

3. Guinea pigs passively sensitized with anti-Friedländer Type B serum and desensitized with the specific carbohydrate remain as resistant to infection with Friedländer's bacillus Type B as undesensitized guinea pigs. Since in this case, at least, it is agreed that type-specific immunity and type-specific hypersensitiveness are due to the same type-specific antibody, a change in anaphylactic response should be accompanied by a change in immune response, provided this change depends on antibody balance.

4. A determination of the antibody content of the serum of sensitized as well as of desensitized guinea pigs by mouse protection tests indicates that a loss of reactivity in desensitized animals cannot be adequately accounted for on the basis of depletion of circulating antibody.

These experiments suggest that hypersensitiveness and resistance are different manifestations of the same antigen-antibody reaction while antianaphylaxis is a state of refractoriness which is due neither to excess of circulating antibody nor to antibody depletion, but is the result of secondary changes the true nature of which is still not definitely established.

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FURTHER STUDIES ON TYPHUS FEVER

ON HOMOLOGOUS ACTIVE IMMUNIZATION AGAINST THE EUROPEAN STRAIN OF TYPHUS FEVER

BY HANS ZINSSER, M.D., AND ATTILIO MACCHIAVELLO,* M.D.

(From the Department of Bacteriology and Immunology, The Harvard Medical School, Boston)

(Received for publication, June 23, 1936)

It appears justified to conclude from the studies of Weigl (1), and from our own work with Castaneda, that active immunization against typhus fever with killed *Rickettsiae* can be achieved, provided a sufficient accumulation of the organisms can be obtained for vaccine preparation. Such mass production of *Rickettsiae* is also an indispensable preliminary for the immunization of horses, donkeys, or goats in the preparation of sera for temporary prophylaxis and for therapeutic application in man.¹ With the murine strains of typhus, as we have reported in preceding papers (2), such *Rickettsia* accumulation has been achieved by a number of methods, all of which depend upon the intraperitoneal infection of rats in which resistance had been reduced by a variety of methods. All such attempts, even the use of preliminary X-ray radiation of the animals (the most satisfactory of the methods employed), have completely failed when the European virus is used. Indeed, our persistent failures in this regard are one of the most convincing reasons for our belief, elsewhere set forth, that the agents of the murine and European infections—so closely related in many of their attributes—are nevertheless distinct and biologically fixed varieties, not permanently convertible one into the

* Guggenheim Fellow.

¹ In this respect, the situation appears to be not unlike that revealed by recent investigations for virus immunization—namely, that for the production of any degree of immunity with killed virus, large quantities of the material are required. It is not impossible that this point of similarity between infectious agents otherwise so far apart in biological attributes may be related to the tendency for intracellular localization which characterizes the pathogenic behavior of both.

other by temporary environmental or experimental manipulations. These matters have been discussed by one of us in another place (3). The important practical consideration which follows from our observations is the fact that for homologous vaccine production with the European *Rickettsiae*, the rat technique is of no value.

In regard to the methods at present available for artificial immunization against the European disease, it appears quite likely that Weigl's phenol-killed louse vaccine is effective in producing a demonstrable degree of immunity. But Weigl's vaccine production necessitates the passage of the virus through large numbers of lice and appears obviously unsuited to the mass immunization desirable for practical purposes in times of epidemic spread. Our own vaccines prepared with the murine strain from rats can be produced in quantity and do protect guinea pigs to a measurable degree from European infection. But the protection is not as complete as similar vaccination against the homologous strain. There is also some protective action (possibly also some therapeutic effect) of the antimurine serum against European infection. Guinea pig experiment has demonstrated this (4). But the few human cases of the European disease treated with this serum do not justify even a preliminary opinion. That there should be some overlapping in these respects is to be expected from the cross immunity observed in animals which have recovered from infection with the respective viruses and from the serological overlapping observed by one of us with Castaneda (5). But in view of the seriousness of the endemic and epidemic problems of the European disease, it would nevertheless be of the greatest practical importance were it possible to develop a method applicable on a large scale and not too complicated, for homologous immunization with the *Rickettsiae* of the European disease.

In endeavoring to find other methods of approach to the problem active and eventually passive immunization against this disease have followed two directions of study: 1. The development of tissue culture vaccine. 2. Serovaccination.

1. Tissue Culture Vaccines

In preparing tissue cultures of the European *Rickettsiae*, we followed with little modification the Maitland method successfully

adapted to this purpose by Nigg and Landsteiner (6). In attempting to improve this method a considerable amount of work has been done which may be briefly summarized, since it may serve to save time for others working along similar lines.

Rickettsiae are extremely selective in regard to the tissue. No growth has been obtained when guinea pig kidney, liver, brain, or heart muscle was substituted for tunica scrapings. Slight growth was obtained with scrapings of the lining cells of the peritoneum. Rabbit tissues have given negative results. Chick embryo likewise.

It was at first thought that guinea pig serum was essential. We have recently found, however, that one part in four of either horse serum or human serum mixed with Tyrode solution before filtration gives even better growth than the guinea pig-serum Tyrode mixtures. It is still to be determined whether there is active metabolism as determinable by oxygen consumption on the part of the guinea pig tissue in horse serum and human serum mixtures. No growth has been obtained in Tyrode solution without serum. Filtered ascitic fluid, substituted for Tyrode solution, if free from bile, gives much heavier growth than the Tyrode-serum mixtures, though individual ascitic fluids vary.

Like Nigg and Landsteiner, we have found that the cultures remain virulent and *Rickettsiae* survive when the flasks are stored without removal of the stoppers. In this manner, a tissue culture of the Mexican strain has been found virulent after 7½ months and a European culture for 4½ months. One of our students, Dr. C. J. Wu, took a sealed murine culture to China, without precautions of refrigeration, and writes us that he obtained successful inoculation on arrival.

The *Rickettsiae* will not grow on tunica tissue heated to 50°C. for 15 minutes. Nigg and Landsteiner (7) reported occasional slight temperature reactions and immunization in guinea pigs after the inoculation of a third generation culture on tissue thus heated, although no *Rickettsiae* could be seen in the material inoculated. They reported the facts without drawing conclusions, but Laigret and others have taken this to indicate the presence of an invisible form of typhus virus, perhaps in cyclic relation with the *Rickettsiae*. We have repeated these experiments a number of times and have in no case obtained either temperature reactions or subsequent immunity in guinea pigs inoculated with materials from the second or third heated tissue culture generations. We venture to suggest that Nigg and Landsteiner's results may have been due to the survival of a few *Rickettsiae* carried over from flask to flask.

Although tissue in such cultures ceases to respire after about 40 hours, as measured in the Warburg apparatus, and the viability of the cells is unlikely to last longer than a week, the most active proliferation of *Rickettsiae* appears to take place after the 4th day. An equilibrium of some kind seems to be established which creates conditions favorable for growth. These conditions, as far as respiration, reaction, and oxidation-reduction potentials are concerned, are being investi-

gated. Assuming the establishment of such an equilibrium, uninoculated tissue cultures were preserved in the incubator for a varying number of days and then inoculated, but no successful cultures were thus obtained.

Efforts are being continued to adapt the Nigg and Landsteiner method to larger quantitative yields by the use of specially designed flasks. It appears that for successful cultivation a definite relationship between the square surface exposed by the fluid and the cubic air space of the closed flasks must be maintained.

For the present studies our methods of tissue culture vaccine production have not varied—except for the frequent substitution of ascitic fluid for Tyrode solution, from the Nigg-Landsteiner procedure.

The source of the culture virus—in three separate isolations, was the material obtained by mincing or by scraping the tunica vaginalis of guinea pigs injected intraperitoneally with large doses of blood and brain mixtures from previously infected animals. Such guinea pigs show, in about 20 per cent of the inoculated, slight scrotal swelling with a few *Rickettsiae* in cell smears. The cultivated strains have been carried on in our laboratory for about 2 years.

The optimum time for the harvesting of such cultures lies between the 8th and the 10th days.

The production of the vaccines has been more or less the same as that employed for the same purpose by Kligler and Aschner (8). Culture flasks are pooled and centrifugalized. The supernatant fluid—in which the numbers of organisms are negligible—is discarded. The bits of tissue are shaken in salt solution containing 0.2 per cent formalin. This removes most of the obtainable *Rickettsiae*. The tissue is again thrown down in the centrifuge and can be further disintegrated by freezing and thawing in a beaker of solid carbon dioxide in ethylene-glycol monomethyl ether—the solution used with the Florsdorf-Mudd apparatus. This is done in pointed centrifuge tubes accompanied by grinding. The ground tissue has so far been impossible on any basis sufficiently accurate to justify description. The amounts of vaccine employed are based on the assumption that the cultures contain approximately equivalent numbers of *Rickettsiae*. 1 cc. represents the yield of about one-fifth of a small (25 cc.) flask, containing a total volume of 2.5 to 3 cc. of culture fluid.

With such vaccines, we have carried out a number of immunization experiments. All vaccine injections were subcutaneous. The results of our first fifteen vaccinations were as follows:

One Injection of Vaccine.—A single vaccine injection of 1 cc. Immunity test 1 month later, with brain virus. Temperature touching 104°F. on 8th, 9th, and 10th days. Mild typhus.

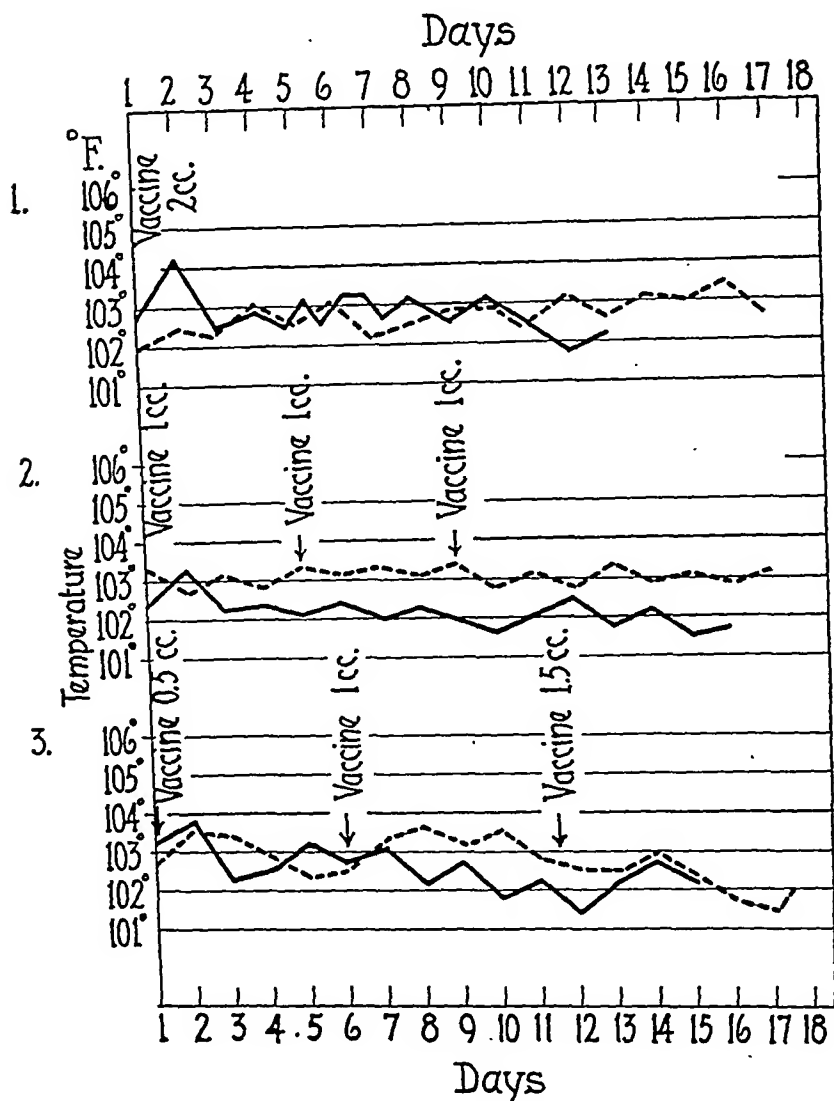


CHART 1. The results of the subcutaneous injection of formalinized tissue culture vaccine in three different types of experiment. The arrows indicate the time of vaccine injections and the amounts. The broken lines represent the results of immunity tests carried out by intraperitoneal inoculation of doses of European brain virus, which gave severe typhus in controls. In curve 1, the immunity test was carried out 33 days after vaccination; in curve 2, 28 days, and in curve 3, 37 days after vaccination.

Two guinea pigs. A single vaccine injection of 2 cc. Immunity test 1 month later. No typhus.

Two Injections of Vaccine.—Five animals were given two vaccine injections, 5 days apart, 1 cc. at each injection. Three of these animals were reinjected after 2 months with heavy doses of brain virus (3 to 5 cc.). All showed mild typhus temperature curves, rising above the critical point of 104°F. for 2, 3, and 4 days respectively. There was apparently a moderate increase of resistance but no immunity.

The other two were similarly reinfected 6 weeks after vaccination and were found immune.

Three Injections of Vaccine.—Eight animals received three injections of the culture vaccine, the intervals varying in the individual experiments from 3 to 4 days. In three of these guinea pigs the dosages were 0.5 cc. at each injection. In others the doses were 1 cc. each and in four animals the doses were 0.5 cc., 1.0 cc., and 1.5 cc. respectively. Intervals between vaccination and immunity tests were from 30 to 38 days. All of these animals were found immune when reinoculated with virus controlled and found potent in normal animals.

Chart 1 shows the results of tissue culture vaccination in a number of animals.

From these experiments it is clear that, as Kligler and Aschner have reported, it is quite possible to obtain effective active immunization in guinea pigs against the classical European virus with formalinized homologous tissue culture vaccines. The experiments further indicate that such immunity may last at least a month and that successful vaccination requires a definite minimum of killed *Rickettsia* material. That a single sufficiently large dose of virus may suffice is indicated in Chart 1. The best results were obtained when three separate injections of vaccine were administered.

2. Serovaccination

While it is always preferable to make use of killed rather than of living infectious agents for immunizations which are intended for eventual application to diseases of man, it has, nevertheless, appeared important to us to explore all the possibilities which offer some hope of practical solution of such large scale problems as those involved in epidemics of European typhus fever.

Our attention was turned to serovaccination by an incidental observation made by one of us, with Batchelder, in 1930 (9). At that time a guinea pig which had been used for neutralization tests of conva-

lescent guinea pig serum against homologous (murine) virus and found fully protected, was reinoculated several weeks later, and failed to exhibit any symptoms of typhus.

Serovaccination has given encouraging results in the past in the immunization of animals against a variety of diseases caused by filtrable virus agents and, within recent years, has been successfully employed in the case of yellow fever, by Sawyer and his collaborators (10). To be sure our own experience with herpes, where fully neutralized virus invariably failed to induce even a partial immunity, was not encouraging. But here, as in poliomyelitis, the immunological problem is complicated by the unsolved difficulties involved in neural transport and in the blood-brain barrier. Between those virus diseases, in which these factors do not enter into consideration, and the *Rickettsia* diseases, there is a considerable immunological similarity, possibly because of the analogous intracellular localization of the responsible infectious agents.

Basing our efforts on these considerations and on the observation cited above, we accordingly proceeded to treat series of guinea pigs with a variety of adjusted mixtures of European typhus virus and protective sera, employing combinations of the two in the following forms.

1. Virus in the form of infectious defibrinated guinea pig blood, or of virulent guinea pig serum, plus convalescent guinea pig serum taken 4 or 5 days after defervescence.

2. Similar defibrinated blood, plus antimurine (heterologous) immune horse serum.

- 3 and 4. Virulent brain suspension with each kind of serum respectively.

- 5 and 6. Tissue culture virus with each kind of serum respectively.

The technique, except in those cases in which brain virus was used, consisted in the preparation of measured mixtures of virus and serum, allowing these to stand for 10 minutes at room temperature, and then injecting intraperitoneally.

A major difficulty encountered in some of these studies was the fact that our original typhus horse, which had been yielding a potent anti-typhus serum, died at this time and all our stocks of active serum contained preservatives. The new horse was delivering a serum of

which the Weil-Felix reaction had not yet exceeded a titer of 1 to 80 (++++), 1 to 160 (+). With a stronger serum it might have been possible to make more accurate adjustments and this, of course, will be done later.

Experiments in which infectious brain was used as virus were on the whole unsatisfactory because, as we have reported in a preceding publication, in order to protect against brain virus in which the injected *Rickettsiae* are presumably intracellular, it is necessary to administer the protective serum in several injections from 24 to 48 hours after inoculation of the virulent material. The probable reasons for this have been discussed elsewhere (4). By following this technique it has indeed been possible, in two experiments of the present series, to immunize guinea pigs by inoculations of brain virus completely neutralized by serum given 24 and 48 hours later as indicated. This method is, however, less certain and more complicated than other procedures to be reported.

Two early experiments gave no information because we used doses of virus so small that even the control animals failed to show the usual symptoms of experimental typhus infection.

As the amounts of virus were increased to points at which the unprotected controls showed late and short lived typhus reactions, the serovaccinated animals, fully protected as regards the vaccination mixtures, did not prove to be immune. (Two experiments.) It was obviously necessary, for successful serovaccination, to make use of mixtures which contained enough virus to produce vigorous experimental typhus in the controls.

In subsequent experiments, therefore, the amounts of virus in the mixtures were so adjusted that the control animals in which normal serum and salt solution were used, reacted with a severe and prolonged experimental typhus. When this technique was employed the results were of two types which we may designate as complete and partial, these terms referring to the degrees of neutralization of the original mixtures.

In the complete experiments, of which there are four to date, the animals receiving the serum-virus mixtures remained entirely normal as far as the usual criteria of experimental typhus are concerned, while the normal serum and salt solution virus controls reacted severely.

Days

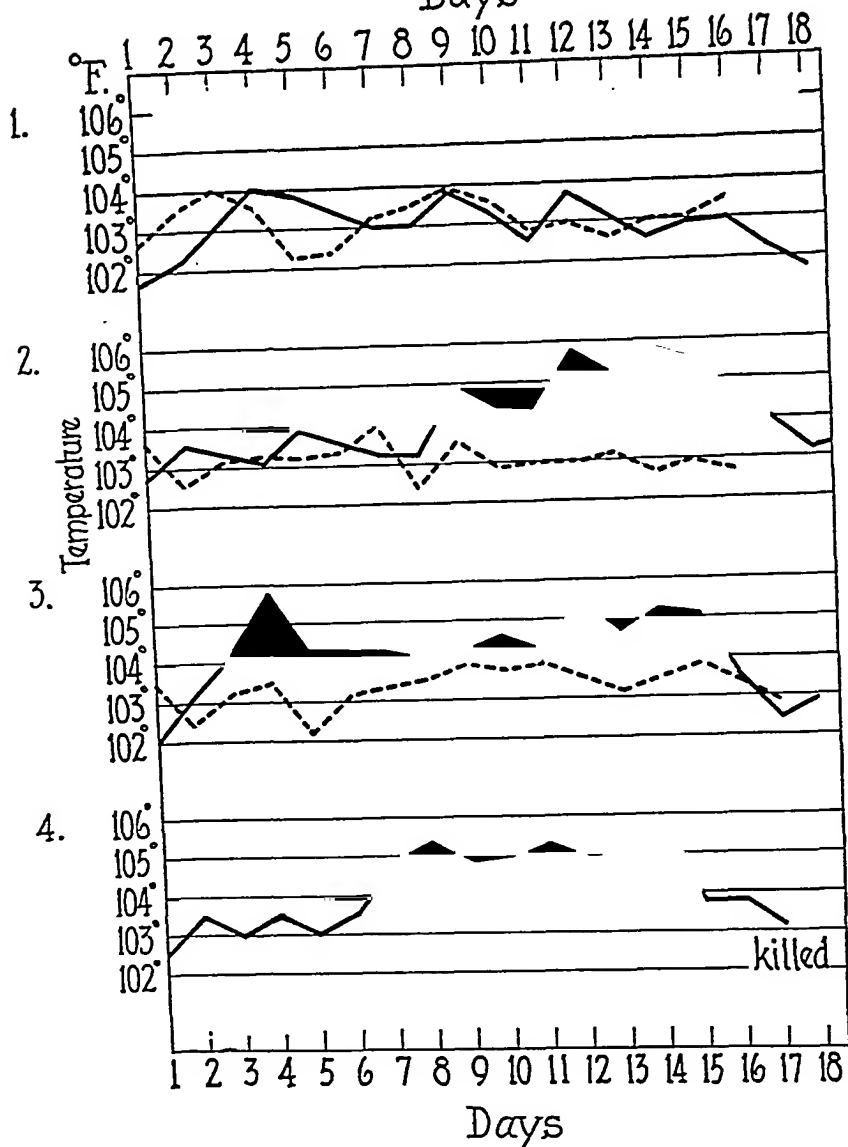


CHART 2. In this experiment serovaccination was carried out on four guinea pigs with mixtures containing, respectively, 0.5 cc. of tissue culture virus (representing about 0.1 cc. of a tissue culture) and 1 cc. of convalescent guinea pig serum, taken from an animal that had passed through a typical European typhus, on the 4th day after defervescence. The mixtures were allowed to stand for 10 minutes at room temperature and injected intraperitoneally.

In this chart, the first curve represents one of three serovaccinated animals. One only is charted, since they were all alike and it was considered particularly important to chart all the controls. In all the curves, the solid blocks represent temperatures above 104°F. The broken lines represent the temperatures after reinoculation with virulent brain emulsion for immunity test. Curve 2 represents the normal serum control. Curve 3 represents the salt solution control. Curve 4 is the control of the brain virus used for immunity test 1 month after the serovaccination.

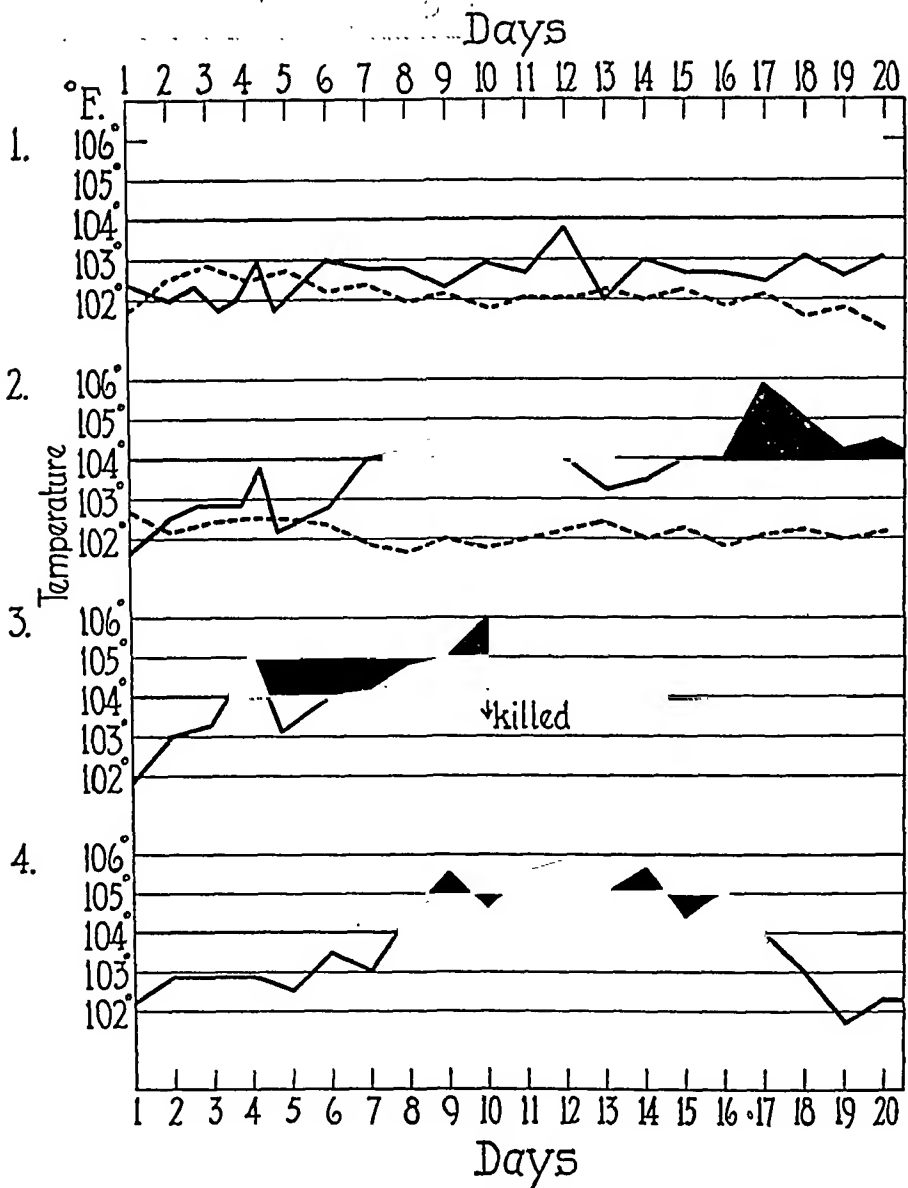


CHART 3. In this experiment, two guinea pigs received 1.5 cc. of virulent defibrinated guinea pig blood mixed with 0.5 cc. of the serum of our second horse, which was being immunized with murine (heterologous) *Rickettsiae* obtained by the X-ray rat method. For reasons stated above, the Weil-Felix titer of this serum did not exceed 1-80 at the time of this experiment. The mixtures were allowed to stand for 10 minutes at room temperature and injected intraperitoneally. One only of the serovaccinated animals is charted, since both were essentially alike in result and it appeared important to chart the controls completely. Temperatures above 104°F. are blocked in black. Broken lines represent the results of immunity tests carried out with virulent brain emulsion of European typhus, intraperitoneally injected 2 months after serovaccination. Curve 2 is the vaccination control in which the virulent blood was mixed with 0.5 cc. of normal horse serum. Curve 3 is the saline control of the virus used for the original vaccination. Curve 4 proves the virulence of the virus employed for reinoculation.

The test animals proved immune when reinoculated with large doses of virus a month or so later. Charts 2 and 3 illustrate such experiments.

In the partial experiments, of which four have been completed at the present time, the relative amounts of virus and of serum in the vaccinating mixtures were such that there was a slight excess of virus over neutralizing power. From the practical point of view these experiments seem to us of considerable importance in that they show that even when complete neutralization is not achieved in the experimentally difficult adjustment between the two reacting factors, the severity of the infection is almost always mitigated, sufficiently to prevent serious infection. A simple table will serve to illustrate these conditions as follows:

Type of serovaccination	Serovaccinated		Normal serum and virus controls	
	Incubation	Duration of fever	Incubation	Duration of fever
	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
1. Virus defibrinated guinea pig blood 2 cc. Immune horse serum 0.5 cc.	16 16	1 2	7 8	10 8
2. Tissue culture virus 0.1 cc. Convalescent guinea pig serum 0.5 cc.	11 11 11	4 2 2	5 5 —	10 8 —
3. Tissue culture virus 0.1 cc. Immune horse serum 0.5 cc.	No fever whatever 11 11	1 1	3 5	10 8
4. Tissue culture virus 0.05 cc. Convalescent guinea pig serum 1 cc.	Two "spikes" of temperature on 4th and 14th days 13 11 11	1 1 4 4	6 3 3	7 10 11

A chart of one of these partial experiments further illustrates these conditions (Chart 4).

It is clear from the experiments recorded that guinea pigs can be immunized against European typhus virus by preliminary injections of mixtures of living virus and protective serum so adjusted that

no experimental typhus results from the injections. It has further been shown that when the perfect balance between the two active factors is not achieved and a moderate excess of virus is present, the serum almost invariably reduces the severity of the infection.

An important question arises in connection with the serovaccination experiments in regard to the fate of the living virus injected in such cases. It is quite conceivable that although such virus is neutralized by the serum with which it is mixed and thereby prevented from causing experimentally determinable infection, it might still remain

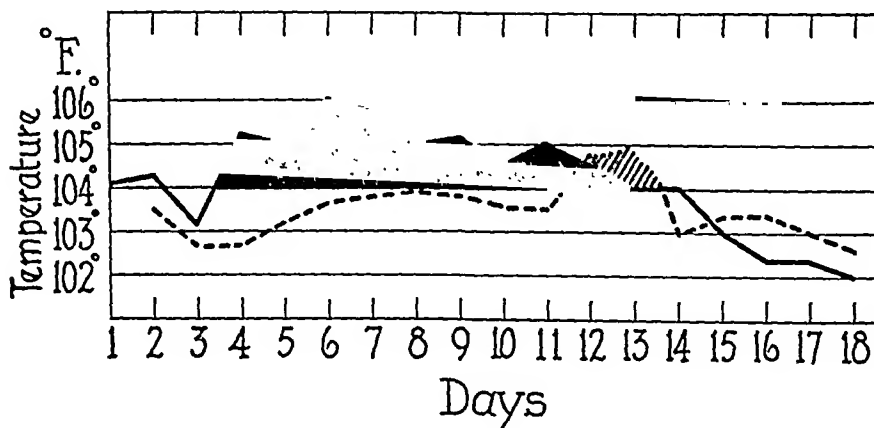


CHART 4. One of the partial serovaccination experiments. The temperature curve represented in broken lines is that of a guinea pig which received, intra-peritoneally, 0.1 cc. of tissue culture virus mixed with 1 cc. of homologous convalescent guinea pig serum. That charted in solid lines represents the reaction in a guinea pig injected with the same amount of virus mixed with normal guinea pig serum.

alive and convert the vaccinated animals into carriers. The importance of such a possibility in connection with any attempts to apply serovaccination to man is obvious. In attempting to approach this problem experimentally we have injected large quantities of brain and blood ($\frac{1}{2}$ brain plus 4 cc. of defibrinated blood) from a successfully serovaccinated animal, killed on the 24th day after the vaccination, into other guinea pigs. The animals so injected showed no reactions whatever. Such tests will require elaboration and, whatever the outcome, it must be borne in mind that in this regard particularly no generalization from guinea pig experiment to man is permissible.

DISCUSSION

Although the manner of transmission and the rôles played by insects and by rodents in the dissemination of the typhus fevers is fairly well understood, experience has shown that sanitary measures alone do not, as yet, suffice to control these diseases when circumstances favor epidemic spread. The situation would be materially improved were methods available for specific prophylaxis applicable to infected populations on an adequate scale. In the case of the Mexican (murine) type, vaccine and serum production by methods which depend upon the accumulation of *Rickettsiae* in rats in which resistance has been experimentally reduced have been elsewhere described and are giving encouraging results. But while these murine products exert a certain degree of overlapping protective action on infection with the European virus, their effects in this respect are partial and imperfect.

While, in our opinions, the Weigl louse vaccine is about as effective as any that can be at present obtained, it is quite unlikely that a vaccine of this kind can ever be produced in quantities large enough to be of practical importance in epidemics.

The experiments above recorded have confirmed the effectiveness of formalinized European tissue culture vaccines in the guinea pig experiment. Such vaccines can be produced on a reasonable scale by a trained staff in a well equipped laboratory. Judging from a now extensive experience with injections of formalinized murine vaccines into man, human application of the tissue culture vaccines, with proper sterility control, would seem to be without danger. Our own efforts at present are being exerted toward the development of methods for increased quantity production, in observations on the duration of the active immunity obtained in guinea pigs and on the storage periods for which the vaccines will remain potent. In the meantime, by the now available methods, sufficient amounts of the tissue culture vaccines can be freshly produced for thorough testing in man.

It is not likely, however that, until one succeeds in increasing quantity production and in simplifying *Rickettsia* tissue culture methods, these vaccines can be freshly produced in amounts adequate to meet the serious emergencies of typhus epidemics. It is this consideration which has encouraged our interest in serovaccination. That such a method can be applied effectively is shown by our experiments. It is

equally obvious, however, that as yet it is quite impossible to set up any standard which will insure against moderate excess of virus over serum potency. Nevertheless, the materials for certain types of serovaccination (guinea pig blood and guinea pig convalescent serum) can be made available in considerable amounts with almost no equipment. The method is therefore at least worthy of further study for possible application in the face of destructive epidemics. Our efforts to overcome some of the difficulties involved in the adjustment of virus and serum for serovaccination, by the desiccation of tested virus and virus-serum mixtures *in vacuo* by the Florsdorf-Mudd method, have thus far failed. Such failure has been due to the deterioration of the European virus under these conditions, within 50 days, a period during which the murine virus, similarly desiccated, appears to retain full potency. These experiments are being continued.

SUMMARY

1. Guinea pigs can be actively immunized against European typhus fever with homologous formalinized *Rickettsia* tissue cultures, provided sufficient amounts are injected. The method is suggested for practical application in man.
 2. Serovaccination against European typhus fever can be successfully applied to guinea pigs by a variety of methods, the simplest of which consists of the injection of mixtures of virulent defibrinated guinea pig blood and convalescent guinea pig serum taken from 3 to 5 days after defervescence. Similar results can be obtained with mixtures in which tissue culture virus, either with convalescent guinea pig serum or with antimurine horse serum, is used. There is no indication so far that such animals become carriers.
- Possible application of these methods to typhus epidemics is discussed.

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STUDIES ON THE MECHANISM OF IMMUNITY IN TYPHUS FEVER*

I. RICKETTSIA PROWAZEKI IN THE DIFFERENT STAGES OF THE TYPHUS LESION

By M. RUIZ CASTANEDA, M.D.

(From the Department of Bacteriology and Immunology of The Harvard Medical School, Boston)

PLATES 38 TO 40

(Received for publication, June 23, 1936)

The demonstration of histopathological changes in typhus fever was first presented by Fraenkel who, in 1914, described microscopic lesions in the skin of typhus patients. Prowazek, Fraenkel, Otto and Dietrich, Spilmeyer, and others found lesions in the central nervous system and in other organs. Such lesions were extensively studied by Wolbach, Todd, and Palfrey, whose work is a classic on the subject (1).

The fundamental picture of a typhus lesion, regardless of its location, consists in a cellular infiltration around blood vessels whose endothelium has been damaged. Such damage depends on the actual multiplication of the typhus virus in the cells of the endothelium of the affected vessel.

The finding of *Rickettsia prowazeki* in the typhus lesion has been a matter of considerable difficulty. After the work of Prowazek and da Rocha-Lima (1916) it was considered necessary to find intracellular organisms in mammals, similar in appearance to those found in the cells of the infected louse.

The first direct demonstration of *Rickettsiae* in the cells of typhus infected men and guinea pigs was presented by Wolbach and co-workers (1920-22). They showed that the swollen cells of the endothelium of small vessels contained numerous organisms morphologically resembling the intracellular *Rickettsiae* of von Prowazek and

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da Rocha-Lima. A second important demonstration was presented by Mooser (2), who found, in the tunica vaginalis of guinea pigs infected with Mexican typhus, cells of mesothelial origin parasitized by a large number of organisms identical with the *Rickettsia prowazeki*. Pinkerton (3) duplicated this finding in guinea pigs infected with European typhus. The last mentioned author (4) also found, after considerable search, *Rickettsia* bodies in the cells of the small vessels of the scrotal sac. From the cytological changes observed by Mooser in the tunica vaginalis of typhus infected guinea pigs (5), Mooser and Dummer (6) inferred that *Rickettsiae* were digested by polymorphonuclear leucocytes, which in turn were then taken up by the mononuclears. The nodules formed by the accumulation of the latter cells represent according to these authors a healing stage of the typhus lesion.

As a direct application of these findings Zinsser and Castaneda (7) developed a method by which *Rickettsiae* have been cultivated within mesothelial cells in the peritoneal cavity of rats, from which large quantities of organisms were obtained. The purified suspensions of *Rickettsiae* have been successfully used in active immunization against typhus fever. With rich vaccines a serum has been prepared in horses which has prophylactic and therapeutic value (8).

The purpose of this paper is to present a systematic study of the lesions produced in the skin of guinea pigs by intradermal and intracardial inoculation of the typhus virus. The position of the *Rickettsia* bodies in the lesions is followed at the different stages of the tissue reactions.

Material and Technique

1. The virus used for inoculum was obtained from the tunica vaginalis of guinea pigs and from the peritoneum of rats infected with Mexican typhus.

The tunica vaginalis of guinea pigs killed on the 7th day of the disease was washed in a 1/10 dilution of guinea pig serum in saline. The washings were centrifuged at low speed for a few minutes and the cell-free supernatant inoculated as soon as possible.

Rats were submitted to short wave X-ray radiations, then inoculated with typhus virus, and were killed on the 5th day after this injection (7). The peritoneal cavity was washed with saline or diluted guinea pig serum. The washings were centrifuged at low speed and the supernatant fluid, usually very rich in *Rickettsiae*, was diluted before inoculation. A rough titration of the rat inoculum

was made by smearing a standard loop on a measured surface. The fixed smears were stained and the number of organisms counted per oil immersion field.

The inoculum for the intracardial inoculations was prepared in the same manner except for using an isotonic solution of sodium citrate to prevent clotting of the peritoneal exudate.

2. White adult guinea pigs were shaved on the sides and inoculated intradermally with 0.2 cc. of the inoculum. The intracardial injections were made under ether anesthesia.

3. The *Rickettsia* bodies were demonstrated by staining the preparations according to the following methods.

(a) Smears from scrapings of the tissues were treated by the methylene blue-safranin method described elsewhere (9).

(b) The extracellular *Rickettsiae* were readily stained with the following mixture.

Phosphate buffer solution pH 7.6 (same as that used in (a))	50
Formalin (40 per cent)	2.5
1 per cent alcoholic solution of methyl violet	1

The smears were fixed by heat and stained for 1 to 5 minutes and washed.

(c) Staining sections. The tissues are fixed in Regaud's fluid (one part of formalin to five parts of a solution containing 2.5 per cent potassium dichromate and 1 per cent sodium sulfate) for 24 hours, washed, and imbedded in paraffin in the usual way. Sections cut as thin as possible are stained by a Giemsa solution prepared as follows: To a 2.5 to 5 per cent solution of formalin in distilled water sufficient acetic acid is added to bring the pH to about 5.5. Usually, for neutral formalin, 0.1 cc. of a 1/10 solution of glacial acetic acid is enough for 100 cc. of the solution. The acidity of commercial formalin is sometimes such that no addition of acetic acid is required. Add to 100 cc. of the acidified formalin solution 2 cc. of Giemsa solution and stain the sections for periods of time lasting from 6 to 18 hours. After staining, the sections are rinsed with water and the excess moisture wiped off. They are then rapidly dehydrated with absolute alcohol followed by xylol and mounted in cedar oil.

In our experience, many trials are often necessary in order to obtain good staining solutions.

The acid Giemsa solutions are stable and for this reason one bath is enough for the entire period of staining. This formalinized Giemsa acts, by its acidity, as a restraint upon the tendency of ordinary Giemsa to stain the tissues blue. The formalin also serves as a mordant for the *Rickettsia* bodies which are easily detected in the faintly stained cytoplasm. An excess of acidity increases the red staining with poor results.

The sections stained for 6 hours show *Rickettsia* bodies stained blue. The nuclei of the cells stain blue. Connective and muscle tissues are red and the blood elements retain their ordinary reactions. The granulations of mast cells take a brilliant purple. The longest periods of staining reveal a similar picture but the blue effect becomes deeper and purplish.

If necessary, the sections may be restained, after washing with xylol and alcohol, by a 2 hours immersion in the formalinized Giemsa solution.

Microscopic Lesions Produced by Intradermal Inoculation of Mexican Typhus Virus

With the exception of a few guinea pigs which did not react after the intradermal inoculation of tunica washings from typhus guinea pigs, the rest of the animals, treated with guinea pig or rat material, showed reactions at the site of inoculation, which varied in intensity according to the numbers of *Rickettsiae* inoculated. A moderate inoculum, as in the case of guinea pig tunica scrapings, produces an immediate congestive reaction which invariably fades in the next few hours. However, from 24 to 48 hours later the local inflammatory reaction becomes definitely conspicuous doubtless due to its progress, which continues to develop in intensity for the subsequent 2 to 4 days. This is followed by an induration of the skin, which may last for about 2 weeks after the inoculation. In mild cases the lesions may disappear within a week, but in strong reactions the inflamed skin may become ulcerated in the center, which retards the healing of the lesions. The size of the swollen and congested wheals varies from 1 to 2.5 cm. in diameter.

When the inoculum is prepared from typhus infected rats, the reactions are usually of the more intense type and are apparent 24 hours after the inoculation.

The lesions described above have been found in a large number of guinea pigs. Such lesions have not been observed after the inoculation of brain or serum from typhus infected animals.

General Symptoms.—In addition to the local reaction at the site of the injection of typhus virus, the guinea pigs show an elevation of temperature which may appear as early as 48 hours after the inocula-

tion. The temperature is usually moderately elevated and maintained for short duration. A few animals had fever which lasted over a week, while others did not show fever at all, particularly those presenting mild skin reactions.

Microscopic Findings

The peritoneal washings of typhus infected rats were the inoculum used for the production of the skin lesions described in this study.

White guinea pigs were shaved on the sides and inoculated intradermally in several different places, usually four to six, and the site of cutaneous inoculation was removed¹ at intervals of 24 hours. Two guinea pigs were usually prepared for each series of skin lesions. Part of the removed skin was scraped, smeared, and stained by the methylene blue-safranin method for direct examination. The rest of the skin was fixed in Regaud's fluid and prepared by the usual methods for subsequent sectioning and staining with the formalinized Giemsa solutions according to the technique already described.

Results of the Direct Examination.—The examination of the smears made from scrapings of the removed skin showed few or no *Rickettsiae* 24 to 48 hours after inoculation. After 72 to 96 hours, intracellular and extracellular *Rickettsia* bodies were found in relatively large numbers. Polymorphonuclear leucocytes, some of which contained phagocytized *Rickettsiae*, were also found, an observation frequently seen in smears from the tunica of guinea pigs and the peritoneum of rats infected with typhus. From the 5th day on, the finding of *Rickettsiae* in the scrapings of the skin lesions was more and more difficult until their complete disappearance on about the 8th day.

Examination of the Sections.—At the site of the inoculation, the epidermis is interrupted and its place is infiltrated by a considerable number of polymorphonuclear leucocytes. This focal infiltration may be seen as early as 24 hours after the inoculation and it gradually increases in intensity to be subsequently displaced by the usual reparative processes. In other skin lesions studied, no local infiltration appeared in the epidermal region. There is no evidence of *Rickettsia* bodies in the zone of infiltrating polymorphonuclears at the epidermis. These cells appear mostly degenerated.

The corium, subdermis, and usually the muscularis, show various

¹ Under ether anesthesia.

degrees of inflammatory response. The type of reaction in the first 48 hours manifests considerable cytological variation. This may be in the form of a moderately diffuse infiltration by mononuclear phagocytes interspaced by few polymorphonuclear leucocytes or the diffuse infiltration may be characterized by preponderance of polymorphonuclear cells. There is some extravasation of red cells particularly in the subcutis. As early as 24 to 48 hours after the inoculation, there is evidence of swelling of the endothelial lining of the capillaries and small blood vessels, in which not infrequently one may find cells packed with *Rickettsiae*. Around larger vessels, there is a perivascular infiltration of mononuclear phagocytes.

As the lesions progress, the perivascular infiltrations become more intense, with prevalence of mononuclear type of cells. 48 to 72 hours after the inoculation of the virus, the lymphatics may be seen occluded by a delicate reticulum of fibrin containing polymorphonuclear leucocytes. Some lymphatics are simply dilated and show fibrin in the lumina. The blood vessels present changes due to marked swelling of the endothelium and early thrombus formation. The small arteries may be occluded by the swollen and vacuolated cells of the intima.

On the 3rd to 4th day, the infiltration by mononuclear phagocytes is conspicuous in all layers of the skin, particularly in the lower portion of the corium. When the vessels are cut transversely the perivascular infiltration displays nodular formations of various sizes, depending on the caliber of the vessels involved. The walls of the vessels within the nodules may or may not be damaged. When one searches for *Rickettsiae* in such nodules it may be possible to find the organisms within the endothelial cells of a small vein, but it is more frequent to observe the typical Mooser's cells outside the larger vessels. When serial sections are followed, such cells can be traced to the capillaries within the nodular formation.

The best places to look for *Rickettsia* cells is the capillary wall, which is easily followed by a swollen endothelial lining. The cells are usually found packed with *Rickettsia* bodies in the same way as those seen in smears from the tunica vaginalis of typhus infected guinea pigs as described by Mooser. There is no cellular reaction around such cells at this stage. Amidst the dense mononuclear phagocytic infiltration of the subdermis, cells containing *Rickettsiae*

are frequently found, also small vessels with their lumen occluded by a swollen cell filled with *Rickettsiae*.

The criteria for the diagnosis of *Rickettsiae* in our sections have consisted either in the finding of typical Mooser's cells or the cells of blood vessels showing the organisms sufficiently clearly to exclude the possibility of confusion with granular material of cellular origin.

We have been unable to obtain any evidence of *Rickettsia* bodies in arteries and lymphatic vessels.

It is interesting to note that one frequently finds veins occluded to variable degrees by mural thrombi. Such thrombi are formed by fibrin lined by a layer of endothelium. The search for *Rickettsiae* in such veins has not been successful.

On the 4th and particularly on and after the 5th day subsequent to inoculation there are recurrences of polymorphonuclear infiltration. Here and there one may see small foci of these cells on the 4th day. When observed under oil immersion, one frequently finds such foci adjacent to swollen cells of the capillaries.

Some of the infiltrations around small vessels may show a prevalence of polymorphonuclear leucocytes or may be entirely formed by these cells. In such cases the vessels are found greatly degenerated.

Interspaced areas of polymorphonuclear leucocytes of considerable size may be found within the mononuclear phagocytic infiltration of the subdermis. (Sometimes one may find veins surrounded by mononuclear phagocytes showing within their lumina a number of polymorphonuclears adhering to the walls of the vessels.)

In searching for *Rickettsiae*, the organisms are more frequently found in places with little or no polymorphonuclear infiltration. With the increase in the polymorphonuclears there is diminution in the numbers of *Rickettsia*-containing cells. When a nodular formation shows an abscess-like appearance *Rickettsiae* are seldom found.

After the 5th day, the mononuclear phagocytic infiltration again predominates and remains as such thereafter. It is difficult to follow the evolution of the nodular formations. However, in view of the considerable phagocytosis of polymorphonuclear leucocytes by macrophages, one may infer that the polymorphonuclears are partly disposed of by phagocytosis and some are returned to the circulation by way of the lymphatics which, as was pointed out, are found to be filled with cells as early as the 4th day after the inoculation.

Intracardial Inoculation of Mexican Typhus Virus into Guinea Pigs

The inoculation of large doses of extracellular *Rickettsiae* by intracardial route has produced the following results. The removal of fragments of skin at daily intervals, as well as the study of the organs at various intervals after inoculation has given relatively few positive findings. On the 3rd day after inoculation it was found, in one animal, that a fragment of the skin contained capillaries with swollen cells filled with *Rickettsia* bodies. The involved vessels were found immediately under the epidermis. In the same animal a small vein was also found in the subdermis that contained *Rickettsiae* within its endothelial cells. No cellular infiltration was noticed around or near such vessels. In another animal, which was killed on the 3rd day, a number of infected cells was found in the veins of the polar fat of the testis. The organisms were rather large and there was some infiltration by mononuclear phagocytes and polymorphonuclears in the intervascular spaces.

Up to the present time we have not made a more intensive search for *Rickettsiae* in the organs of typhus infected guinea pigs, but according to the results obtained in the few animals thus far studied, the detection of *Rickettsiae* outside of the tunica, peritoneum, and local skin lesions, is a difficult task.

SUMMARY

This study of the lesions produced in the skin of guinea pigs inoculated intracutaneously with Mexican typhus virus, shows that there is an early polymorphonuclear response at the point of inoculation. As early as 24 hours after the virus is given, a mononuclear phagocytic infiltration, which is more pronounced around the larger vessels of the corium, vascularis, and muscularis, replaces the polymorphonuclear infiltration. The endothelial cells of capillaries and small vessels swell up, thus partially occluding the lumina. *Rickettsia* bodies are found in the swollen cells, in numbers which remind one of the intracellular *Rickettsiae* of the tunica in typhus infected guinea pigs. The mature Mooser's cells are found in abundance on the 3rd to the 4th day after inoculation. They are found in various positions as follows: (a) in the endothelial cells of capillaries, particularly in places of little or no infiltration; (b) within the mononuclear nodule

formed around the larger vessels and within the dense infiltration of the vascularis (the parasitized cells are usually traced to a capillary wall); and (c) less frequently the organisms are found within the swollen cells of arterioles and small veins. The organisms disappear gradually from the zones of increasing polymorphonuclear infiltrations, suggesting that the presence of such polymorphonuclears is due to the bursting of the infected cells.

In the artificial lesions produced in the skin by the inoculation of considerable numbers of *Rickettsiae*, the tissue reactions are abnormally enhanced. One can see in the same slide different stages of the development of the lesions and their relationship to the infecting agent. The early perivascular infiltration by mononuclear phagocytes does not seem to be related to an actual infection of the endothelial lining by the inoculated virus, but seems rather, when properly controlled, to be primarily due to a nonspecific type of response. The capillaries or small vessels within these infiltrated zones may become parasitized and call forth a polymorphonuclear reaction which may thus transform the cytological picture of the nodule. The subsequent migration of macrophages terminates the histological sequence.

In capillaries apart from areas of cellular infiltrations, the polymorphonuclear reaction is first to appear, when the *Rickettsiae* are liberated from the cells.

One cannot safely generalize from the results observed in an artificial typhus lesion, but in the light of these observations, it is probable that the *Rickettsia* bodies are difficult to find in typhus patients or infected animals because they disappear rapidly from the nodules, or perhaps because some nodules are not necessarily related to an infected endothelium. At any rate, a late typhus lesion is not likely to reveal *Rickettsiae* which most probably have been removed by the early polymorphonuclear invasion.

CONCLUSIONS

The Mexican typhus virus is capable of producing a local inflammatory reaction when injected intradermally into guinea pigs.

Rickettsia bodies are easily found in the skin lesion, particularly in the walls of capillaries, in places of little or no cellular reaction, in the early stages of the disease.

The *Rickettsia* bodies are less frequently seen in places of increasing polymorphonuclear infiltrations.

In the mononuclear phagocytic nodules, characteristic of the typhus lesion, *Rickettsiae* are rarely found. This may perhaps be due to an early destruction by polymorphonuclear phagocytes.

I wish to thank Dr. H. Pinkerton and Dr. V. Menkin for their advice and criticism throughout the course of this work.

Note.—After this paper had been prepared for publication, there appeared an article by Baltazard, M., *Bull. Soc. path. exot.*, 1936, 29, 403. The author observed skin lesions following intradermic injections with murine typhus virus.

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EXPLANATION OF PLATES

Formalinized Giemsa was used to stain all sections.

PLATE 38

FIG. 1. *Rickettsiae* in a capillary from the dermis of a guinea pig 72 hours after the intracardial injection of Mexican typhus virus. Notice the absence of cellular infiltration. $\times 2000$.

FIG. 2. Bursting Mooser cell in a capillary. Local skin lesion 4 days after intradermal inoculation with Mexican typhus. $\times 2000$.

FIG. 3. Perivascular infiltration with prevalence of mononuclear cellular infiltration. Notice *Rickettsiae* in the wall of an afferent capillary to the vein. 4th day skin lesion produced by intradermal inoculation. $\times 2000$.

PLATE 39

The photomicrographs of Figs. 4 to 6 and those from Figs. 7 and 8 were made from sections of the skin lesions produced by intradermal inoculation of Mexican virus.

FIG. 4. Perivascular infiltration by mononuclear phagocytes on the 4th day. Capillaries containing *Rickettsia* bodies are marked with arrows at the periphery of the infiltration. $\times 600$.

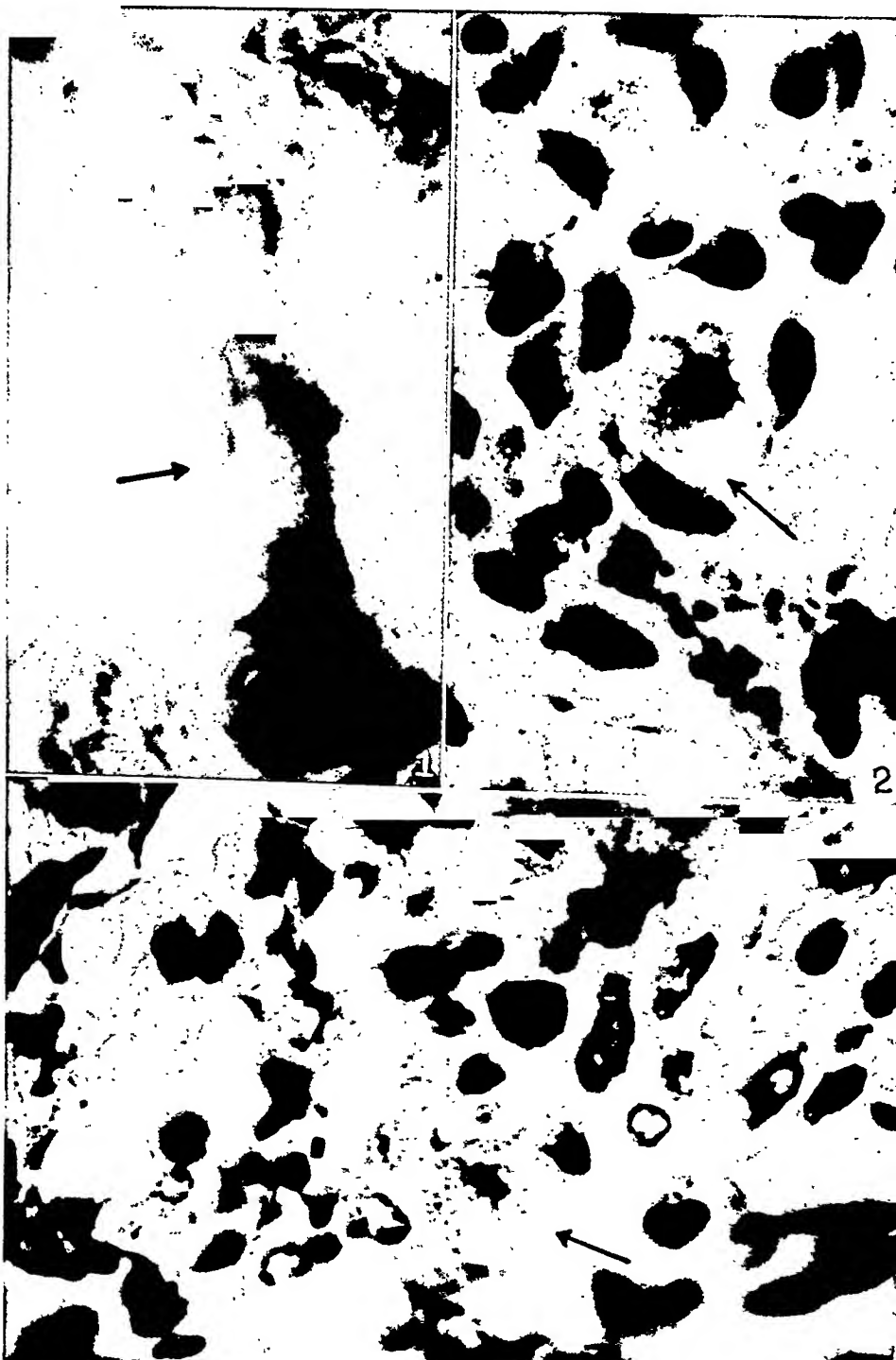
FIG. 5. Nodule on the 5th day. Notice increase in polymorphonuclear leucocytes. The small vessel (arrow) is swollen and occluded. No *Rickettsiae* were found. $\times 400$.

FIG. 6. Nodule on the 6th day. Notice the damage of the blood vessels and cellular elements. There is phagocytosis of polymorphonuclear leucocytes by macrophages. $\times 400$.

PLATE 40

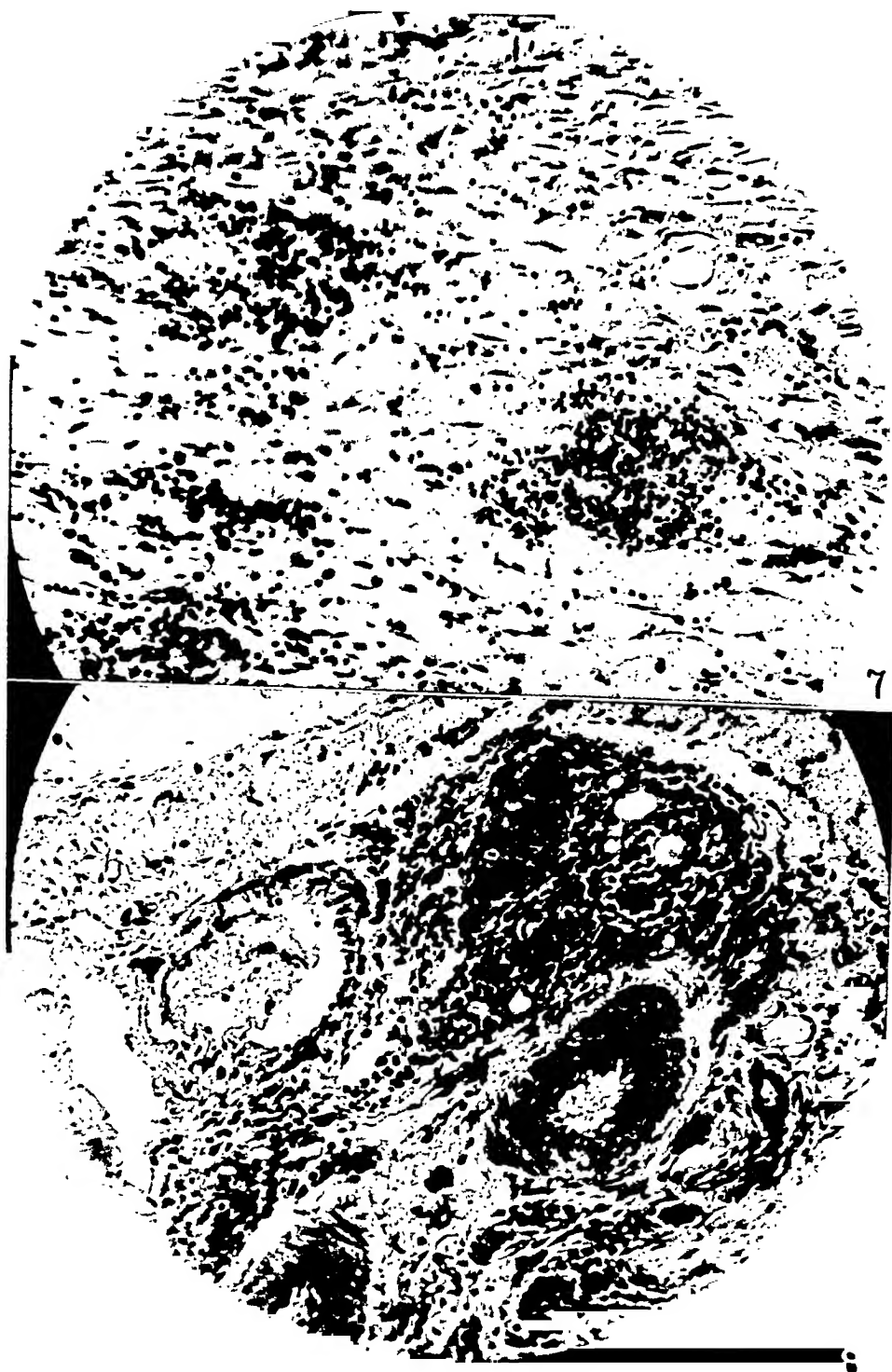
FIG. 7. Mononuclear phagocytic nodules in the subdermis. 10th day lesion. No *Rickettsiae* were found. Low power magnification.

FIG. 8. 4th day lesion. Dense infiltration by mononuclear phagocytes and polymorphonuclears. Notice large vein with mural thrombus of fibrin covered by proliferating endothelium. No *Rickettsiae* were found in the cells of this vein.





(Castaneda: Mechanism of immunity in typhus fever. 1)



(Castaneda: Mechanism of immunity in typhus fever. 1)



(Castaneda: Mechanism of immunity in typhus fever. 1)

STUDIES ON THE MECHANISM OF IMMUNITY IN TYPHUS FEVER*

II. ALLERGIC AND TOXIC REACTIONS PRODUCED WITH RICKETTSIA PROWAZEKI

By M. RUIZ CASTANEDA, M.D.

(From the Department of Bacteriology and Immunology of The Harvard Medical School, Boston)

(Received for publication, June 23, 1936)

In a former paper¹ we reported on the reactions produced in the skin of normal guinea pigs after the intradermal inoculation of Mexican typhus *Rickettsiae*. The local reactions resulting from such treatment afforded an opportunity to follow the agent of typhus fever through the different stages of the development of the lesion. The possibility that *Rickettsiae* disappear from the typhus lesion because of an early phagocytosis by polymorphonuclear leucocytes was discussed.

In the present paper we are reporting certain results which follow the intradermal inoculation of live and killed Mexican *Rickettsiae* into typhus immune animals. In addition, toxic reactions produced in the skin of normal men and guinea pigs upon the injection of formalinized *Rickettsiae* are described.

Materials and Technique

The material referred to as live *Rickettsia* suspensions, was prepared according to the methods reported previously.¹ The suspensions of washed *Rickettsiae* were obtained from rats exposed to X-ray radiation and subsequently infected with typhus virus. The animals were killed on the 5th day after the inoculation and the peritoneum washed with an isotonic solution of sodium citrate. These washings were mixed with an equal amount of normal guinea pig serum which had been diluted 1/3 in saline. The serum prevents the rapid deterioration of the typhus virus which occurs when it is suspended in saline only. The cells and

* This work was partially supported by a grant from the DeLamar Mobile Research Fund.

¹ Castaneda, M. R., *J. Exp. Med.*, 1936, 64, 689.

detritus were removed by low speed centrifugation, following which the supernatant fluid was centrifuged at high speed and this sediment was resuspended in a 1/6 dilution of normal guinea pig serum. This product was again centrifuged at high speed and the sediment resuspended in a small volume of saline. Smears stained with methyl violet revealed *Rickettsiae* in numbers which were usually over 1000 organisms per oil immersion field. Part of the washed *Rickettsiae* was diluted in guinea pig serum in order to obtain suspensions of about 20 live organisms per oil immersion field. The remainder of the emulsion was heated at 60°C. for 1 hour. The formalinized *Rickettsia* suspensions were prepared in the same manner except for the use of 0.2 per cent formalin in citrate and in salt solutions instead of the diluted serum.

The live or killed *Rickettsia* suspensions were injected intracutaneously into the shaved skin of white guinea pigs in doses of 0.2 cc.

Intradermal Inoculation of Live Rickettsiae into Typhus Immune Guinea Pigs

The results obtained by inoculating immune guinea pigs with tunica washings from typhus infected guinea pigs were not uniform. However, the richer material prepared from the peritoneum of typhus infected rats, produced reactions with consistent regularity. The hypersensitivity of the skin to the proteins contained in the inoculum was controlled by the intradermal injection of diluted guinea pig or rat serum. Table I indicates the various types of reactions produced by the intradermal inoculation of Mexican typhus virus into immune guinea pigs. Each of the first three animals received an inoculum prepared from a typhus infected guinea pig. The rest received virus derived from typhus infected rats.

Many experiments, similar to those presented in Table I, demonstrated that guinea pigs which have recovered from European or Mexican typhus, respond to reinoculation with the Mexican virus by a local inflammatory reaction at the site of the intradermal injection. The reactions appear in 24 to 48 hours after inoculation, reach their maximum intensity by the 48th hour, and fade from the 3rd to the 5th day. The center of the inflamed zone may become ulcerated, presenting the appearance of a tuberculin reaction. When this occurs the formation of a scab retards the healing of the injured skin.

If the reactions shown by both normal and immune animals after the intracutaneous injection of Mexican virus be compared it will be observed that the reactions appear earlier and fade sooner in the im-

TABLE I
Intradermal Inoculation of Live Rickettsiae into Typhus Immune Guinea Pigs

Guinea pig No.	Type of virus immunization	Date of the skin test	Day following the test							Inoculum		
			2	3	4	5	6	7	8		9	
4-74	Mexican typhus Aug. 4, 1935	1935 Oct. 21	±	+	±	-	-				Guinea pig tunica	
			-	-	-	-	-	-	-	-	" " serum 1/5	
			-	-	-	-	-	-	-	-	" " tunica	
4-95	European typhus Aug. 10, 1935	"	±	+	+	±	-				" " " serum 1/5	
			-	-	-	-	-	-	-	-	" " tunica	
			-	-	-	-	-	-	-	-	" " " serum 1/5	
4-77	Normal	"	±	+	+	+	+	+	+	Scab	" " tunica	
			-	-	-	-	-	-	-	-	" " " serum 1/5	
			-	-	-	-	-	-	-	-		
4-27	Mexican typhus Oct. 21, 1935	Nov. 18	+	+	±	Scab					Rat peritoneal exudate	
			+	-	-	-					" " serum 1/5	
			-	-	-	-					" peritoneal exudate	
2-93	European typhus July 21, 1935	"	+	+	+	Scab					" " serum 1/5	
			±	-	-	-					" peritoneal exudate	
			-	-	-	-					" " serum 1/5	
1-36	Normal	"	±	+	+	+	+	+	+	+	+	" peritoneal exudate
			±	-	-	-	-	-	-	-	-	" " serum 1/5
			-	-	-	-	-	-	-	-	-	

±, very slight congestion at the site of the inoculation.

+, zone of congestion 10 mm. in diameter.

++, moderate inflammation of the skin which may become ulcerated.

+++ and +++++, stronger reactions.

TABLE II
Intradermal Inoculation of Mexican Typhus Virus into Guinea Pigs at Various Intervals of Time after the First Typhus Inoculation

Guinea pig No.	Type of virus and days after first injection	Date of the skin test	Day following the test									Inoculum	
			2	3	4	5	6	7	8	9			
1936													
3-40	Normal	Feb. 13	-	+	++	++	++	++	++	+	Guinea pig tunica		
3-35	Mexican	"	-	-	-	-	-	-	-	-	"		
1-59	"	"	±	-	-	-	-	-	-	-	"		
3-27	"	"	++	±	-	-	-	-	-	-	"		
1-56	"	"	++	±	-	-	-	-	-	-	"		
1-74	"	"	++	++	±	-	-	-	-	-	"		
3-52	Normal	Mar. 5	-	++	++	++	++	++	++	Scab	"		
3-48	Mexican	"	±	±	+	±	-	-	-	-	"		
3-79	"	"	+	±	-	-	-	-	-	-	"		
3-76	"	"	±	-	-	-	-	-	-	-	"		
3-42	"	"	-	+	±	-	-	-	-	-	"		
3-35	"	"	±	+	-	-	-	-	-	-	"		
3-45	European 16	"	-	+	+	-	-	-	-	-	"		
3-34	"	"	±	++	+	-	-	-	-	-	"		
3-52	Normal	"	++	++	++	++	++	++	++	Scab	Rat peritoneal exudate		
3-48	Mexican	"	±	+	+	±	±	-	-	-	"		
3-79	"	"	++	++	++	+	±	-	-	-	"		
3-76	"	"	+	+	+	-	-	-	-	-	"		
3-42	"	"	++	-	-	Scab	-	-	-	-	"		
3-35	"	"	++	++	+	"	-	-	-	-	"		
3-45	European 16	"	++	++	±	-	-	-	-	-	"		
3-34	"	"	++	++	++	Scab	-	-	-	-	"		

immune animals. The lesions are more severe in the normal guinea pig. The inoculation of large doses of virus produces reactions which are usually of the same intensity in animals immune to both types of typhus. However, it has been observed that, when a moderate inoculum was used as a test dose, the European typhus immune guinea pig develops stronger reactions than the Mexican typhus immune animals.

After a first inoculation with typhus virus, variations in the reactions to the intradermal test depend upon intervals between the injections and the quantity of the inoculum. Table II summarizes the results obtained in two series of Mexican typhus immune guinea pigs tested at periods of time ranging from 5 to 69 days after the first typhus infection. The second series were tested at the same time with tunica washings and rat peritoneal washings. Two European typhus immune guinea pigs are included in the second series. The negative results of the control injections with guinea pig and rat sera are omitted from the table in order to save space.

Table II indicates that typhus immune guinea pigs begin to react with an accelerated type of reaction after the 15th day from the first administration of the virus. During the first 10 days after the infection the animals tested with a strong inoculum may react in a manner similar to that produced by a mild inoculum in normal animals. There is a short period of time, from the 10th to the 15th day, in which the animals develop no significant reactions at the site of the test inoculation.

The accelerated skin reaction shown by the immune animals has been produced several times in the same animal at intervals of time varying from 5 to 30 days between the tests. In case of repeated tests with rat material, care was taken to avoid confusion with the reactions due to the development of dermal sensitivity to rat protein.

Survival of the Typhus Virus in the Local Lesion Produced by Intradermal Inoculation into Typhus Immune Guinea Pigs

The skin, at the site of the inoculation of Mexican *Rickettsiae* into typhus immune guinea pigs was removed at 24 hour intervals. The removed skin was emulsified in saline and inoculated intraperitoneally into normal male guinea pigs. The results of such experiments, as

indicated in Table III, have shown that the virus remains alive in the skin of the immune animals for 72 hours after the inoculation. However, in the Mexican typhus immune 1-84, in which guinea pig tunica washings were inoculated, the virus disappeared sooner than in the similarly treated European immune animal.

TABLE III

Survival of Mexican Typhus Virus in the Local Skin Lesion Produced by Intradermal Inoculation into Immune Animals

Guinea pig No.	Type of virus immunization and days after inoculation	Date of skin test	Inoculum	Removal of skin	Results of the intraperitoneal inoculation of the removed skin into male guinea pigs
		1936		hrs.	
1-66	European 28 days	Jan. 4	Guinea pig tunica	24 48	Guinea pig 2-00—Positive with scrotal swelling on the 7th day Guinea pig 1-60—Positive with scrotal swelling on the 6th day
1-84	Mexican 36 days	" "	" "	24 48	Guinea pig 1-33—Positive with scrotal swelling on the 13th day Guinea pig 1-59—Negative
3-91	European 43 days	Mar. 5	Rat peritoneal exudate	48 72	Guinea pig 3-71—Positive with scrotal swelling on the 6th day Guinea pig 3-70—Positive with scrotal swelling on the 7th day
3-38	European 73 days	Apr. 2	" "	96	Guinea pig 9-6—Negative
4-87	Mexican 26 days	" "	" "	48 72	Guinea pig 9-7—Positive with scrotal swelling on the 9th day Guinea pig 8-4—Positive with scrotal swelling on the 13th day

Microscopic Examination of the Skin at the Site of the Inoculation of Typhus Virus into Immune Guinea Pigs

The skin at the site of the reaction was removed at intervals of 24 hours after the intracutaneous inoculation of typhus virus into immune guinea pigs. Sections were prepared and stained by the formalinized Giemsa solution, as described in the previous paper.¹ 24 hours after the inoculation, an infiltration by poly-

morphonuclear leucocytes was noted which was similar to that observed in normal animals. At 48 hours, the corium and vascularis were moderately infiltrated by mononuclear phagocytes and polymorphonuclears. During the following days the infiltration became less marked, contrasting with that seen in normal guinea pigs where the cellular reaction continued to increase after 48 hours. Microscopically, *Rickettsiae* in the skin lesions of immune guinea pigs have not been demonstrated.

TABLE IV

Intradermal Injection of Heat-Killed Rickettsiae into Guinea Pigs (Normal and Typhus Immune)

Guinea pig No.	History	Date of the skin test	Day following the test							Inoculum
			2	3	4	5	6	7	8	
1-75	European immune	1935 Nov. 23	+	++	+	Scab				Rat virus (10 <i>Rickettsiae</i> per field)
			±	±	-	-				Same heated
			±	±	-	-				Rat serum 1/10
1-83	Mexican immune	" "	++	+	±	-				Rat virus (10 <i>Rickettsiae</i> per field)
			±	-	-	-				Same heated
			±	-	-	-				Rat serum 1/10
1-38	Normal	" "	±	+	++	++++	++	+	+	Scab
			±	-	-	-	-	-	-	Rat virus (10 <i>Rickettsiae</i> per field)
			±	-	-	-	-	-	-	Same heated
										Rat serum 1/10

Intradermal Injection of Killed Rickettsiae into Normal and Immune Guinea Pigs

1. *Heat-Killed Rickettsiae*.—The material used as live *Rickettsiae* inoculum, which has been conveniently diluted in order to obtain a maximum of about 20 organisms per oil immersion field, was heated at 60°C. for 1 hour. The inoculation into normal and immune guinea pigs of the heated and unheated material gave the results recorded in Table IV.

This experiment shows that the heating at a temperature slightly over the minimum necessary to kill the *Rickettsia* bodies, not only

TABLE V
Intradermal Inoculation of Large Doses of Heat-Killed Rickettsiae into Typhus Immune Guinea Pigs

Intradermal Inoculation of Large Doses of Heat-Killed <i>Rickettsiae</i> into Typhus Immune Guinea Pigs											
Guinea pig No.	History	Date of the skin test	Day following the test								
			2	3	4	5	6	7	8		
3-43	Mexican typhus Feb. 13, 1936	1936 Mar. 11	++	+	±	-	-	-			
			+	-	-	-	-	-			
			±	-	-	-	-	-			
			±	-	-	-	-	-			
3-38	European typhus Jan. 8, 1936	"	++	+	+	Scab	-	-			
			+	-	-	-	-	-			
			±	-	-	-	-	-			
			-	-	-	-	-	-			
4-82	Normal	"	++	++	++	++	++	++			
			+	-	-	-	-	-			
		"	±	-	-	-	-	-			
			±	-	-	-	-	-			
		"	±	-	-	-	-	-			
			±	-	-	-	-	-			
* The numbers indicate the <i>Rickettsiae</i> per oil immersion field.											
			Inoculum								
			20 live washed <i>Rickettsiae</i> *								
			1000 heat-killed <i>Rickettsiae</i>								
			100 " "								
			50 " "								
			Mixture of guinea pig and rat sera (1/10)								
			20 live washed <i>Rickettsiae</i>								
			1000 heat-killed <i>Rickettsiae</i>								
			100 " "								
			50 " "								
			Mixture of guinea pig and rat sera (1/10)								

* The numbers indicate the *Rickettsiae* per oil immersion field.

renders the virus innocuous toward the normal guinea pig, but prevents the development of the skin reaction produced in the immune animals by the unheated material.

When rich suspensions of washed *Rickettsiae* are heated for 1 hour at 60°C., the results of the intradermal inoculation into normal and immune animals are as shown in Table V. In this experiment the

TABLE VI

Intradermal Inoculation of Heated and Unheated Suspensions of Formalinized Rickettsiae into Normal and Immune Guinea Pigs

Guinea pig No.	History	Date of the skin test	Day following the test						Inoculum
			2	3	4	5	6	7	
1-81	Mexican typhus Nov. 29, 1935	1936 Jan. 27	++	++	+	-	-	-	Guinea pig tunica washing
			+	±	-	-	-	-	Formalinized <i>Rickettsiae</i> (1000 per field)
			+	±	-	-	-	-	Same heated 70°C.
			±	±	-	-	-	-	Rat serum diluted 1/10 in 0.01 per cent formalin
			±	-	-	-	-	-	Guinea pig serum 1/10
3-32	Normal	" "	±	++	+++	+++	++	++	Guinea pig tunica washing
			++	+	-	-	-	-	Formalinized <i>Rickettsiae</i> (1000 per field)
			±	-	-	-	-	-	Same heated 70°C.
			±	-	-	-	-	-	Rat serum diluted 1/10 in 0.01 per cent formalin
			±	-	-	-	-	-	Guinea pig serum 1/10

effect of 20 live washed *Rickettsiae* per oil immersion field is compared with that of various suspensions of heat-killed *Rickettsiae* containing 1000, 100, and 50 organisms per oil immersion field.

As it may be seen in Table V, the heated *Rickettsiae* produced no significant reactions in either normal or immune animals even when injected in relatively large doses.

2. *Formalin-Killed Rickettsiae*. *Toxicity*.—It has been the experience of workers in this laboratory that a concentration of 0.2 per cent formalin (40 per cent) is sufficient to inactivate the virus of typhus in a relatively short time. 24 hours after the organisms are suspended in formalinized saline, the emulsion may be safely used.

Formalinized *Rickettsia* suspensions, containing over 1000 organisms per oil immersion field, were freed from the formalin by centrifugation and resuspension in saline. Part of the emulsion was heated at 70°C. for 40 minutes and the heated

TABLE VII
Titration of the Toxicity of the Formalinized Rickettsiae Suspensions
(Intradermal Inoculation)

Normal guinea pig			Typhus immune guinea pig			Inoculum
24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	
mm.	mm.	mm.	mm.	mm.	mm.	
16*	10*	—	11*	8	—	Unheated formalinized <i>Rickettsiae</i>
12*	7	—	10*	6	—	1000 per oil immersion field
12*	7	—	8	6	—	400 " " " "
10*	6	—	8	5	—	200 " " " "
8	6	—	6	7	—	100 " " " "
8	5	—	8	5	—	50 " " " "
5	5	—	5	4	—	<i>Rickettsiae</i> heated at 70°C. 40 min.
5	4	—	5	4	—	1000 per oil immersion field
5	4	—	4	4	—	400 " " " "
4	—	—	4	4	—	200 " " " "
					—	100 " " " "
						Rat serum diluted 1/10 in 0.01 per cent formalin

* Indicates significant reactions.

and unheated suspensions were injected intradermally into normal and immune guinea pigs. The inoculation of a few live *Rickettsiae* per oil immersion field was included as a control.

In the experiment presented in Table VI the live *Rickettsiae* produced the ordinary reactions in both animals. The unheated formalinized *Rickettsiae* produced a definite reaction in the normal guinea pig. This reaction consisted in a congestion and swelling of the skin at the site of the injection, which appeared at 24 hours and faded 72 hours after the test. The heated *Rickettsiae* produced no reaction in

the normal animal. The typhus immune guinea pig showed a slight reaction of short duration to both heated and unheated *Rickettsiae*. The results of an attempt to titrate what seemed to be a toxic effect of the unheated formalinized *Rickettsiae* are noted in Table VII. In this experiment various dilutions of heated and unheated formalinized *Rickettsiae* were injected into normal and immune guinea pigs. The readings indicate the diameter of the wheals in millimeters.

If one considers the 10 mm. reactions as indicating a definite reaction there appears to be a difference between the readings in the normal and the immune animals to suggest a degree of resistance on the part of the immune guinea pig toward the toxic effect of the unheated emulsions.

The toxicity of the unheated formalinized *Rickettsiae* is apparently due to a heat labile substance contained in the *Rickettsia* bodies. This is shown by the fact that the peritoneal exudate from typhus infected rats has no toxic effect, when the formalinized *Rickettsiae* are removed by high speed centrifugation. The formalinized *Rickettsia* suspensions which have been kept for a month in the ice chest, have been centrifuged and both fractions compared, with the result that the resuspended *Rickettsiae* were still toxic while the supernatants were inactive.

The effects of the formalinized *Rickettsia* suspensions upon human beings have been studied in a small group of persons, some of whom had a definite history of typhus infection.

The unheated formalinized *Rickettsiae* produced, in normal volunteers, a reaction similar to that observed in the normal guinea pig. This reaction consisted in an area of congestion 2 or 3 cm. in diameter, which appeared 24 hours after the intradermal test and faded on the 3rd day. The heated formalinized *Rickettsiae*, injected as a control, produced no reaction.

The injection of formalinized *Rickettsiae* into persons of known typhus history has shown irregular results. One person who had typhus fever in 1929 showed a strong reaction lasting 5 days, to both the heated and unheated *Rickettsiae*. Another immune individual who had typhus fever in 1929 showed no reaction to the heated material and a very slight congestion at the site of the injection of unheated *Rickettsiae*. Some immune people tested by Dr. H. Naterman, in the

Beth Israel Hospital did not react to a suspension of the unheated *Rickettsiae* which had produced a reaction in normal persons tested at the same time. Goodman and Brodie² found persons of known typhus history who reacted to the intradermal inoculation of formalinized *Rickettsiae*, while their controls did not react. In a recent visit to this laboratory, Dr. Brodie informed us that in experiments with a fresh lot of typhus vaccine sent by Dr. Zinsser, he found positive reactions in normal individuals as well as typhus immune persons. Likewise occasionally an individual who had recovered from typhus failed to exhibit the reaction.

DISCUSSION

As was shown in a previous paper, the intradermal inoculation of Mexican typhus *Rickettsiae* into normal guinea pigs produces an inflammation of the skin at the site of injection. This lesion usually begins 48 hours after a moderate inoculum and lasts for about 10 days. *Rickettsia* bodies may be readily found in the swollen cells of the vascular endothelium. The results obtained in the experiments described in the present paper reveal that the typhus immune guinea pig develops a local reaction, similar in its appearance to the lesions observed in normal animals. The reaction in the immune animal usually begins 24 hours after the inoculation, reaches its maximum intensity in from 24 to 48 hours; but contrary to what is observed in the normal guinea pig, the inflammation fades on the 4th or 5th day after the injection. As in the case of the normal animals, the intensity and constancy of the reaction depends on the richness of the inoculum.

The intradermal inoculation of typhus virus into guinea pigs at various intervals of time after the first typhus infection shows that during the first 10 days a rich inoculum produces reactions which resemble the lesions observed in normal animals. From the 10th to the 15th day there is little or no reaction at the site of inoculation. From the 20th day on the skin reactions, with the characteristics described above, are constant in Mexican and European typhus immune guinea pigs. The test has been repeated at intervals of 5 to 30 days in the same animal, with positive results. For this purpose,

² Goodman, C., and Brodie, M., *Proc. Soc. Exp. Biol. and Med.*, 1935, 32, 1332.

guinea pig tunica washings and rat peritoneal washings were alternately used as inoculum in order to avoid the repetition of rat material.

The skin removed from the site of the reaction showed that the virus may be recovered 72 hours after the test in the immune guinea pigs.

The fact that the virus heated at 60°C. for 1 hour loses its property to produce a local reaction, together with the survival of the *Rickettsia* bodies in the immune animals, is an indication that such reactions are stimulated by the living virus and are not the mere result of an allergic response to *Rickettsia* constituents. The beginning of the reaction after a period of incubation of 24 to 48 hours; the acute inflammatory reaction observed in sections of the skin, as well as the variations in the intensity and duration of the lesions according to the intervals between the first infection and the test, suggest that such reactions may be due to a partial state of immunity as is the case in Koch's phenomenon and in the "accelerated takes" in smallpox vaccination of immune persons.

The difference in the intensity of the reactions presented by the Mexican and European immune animals, together with the apparently longer survival of the virus in the European typhus recovered guinea pig, indicate that the latter possesses a weaker state of immunity toward Mexican *Rickettsiae*. This observation is in accord with other immunological and pathological differences found in both types of typhus virus.

The intradermal injection into typhus immune guinea pigs of rich suspensions of heat-killed or formalinized *Rickettsiae*, produces no significant reactions, even in numbers hundreds of times those required to develop a strong reaction with live *Rickettsiae*.

When normal guinea pigs are intradermally injected with suspensions of formalin-killed *Rickettsiae*, there is a congestion and swelling of the skin at the site of the injection, which appears 24 hours after the test and usually lasts 48 hours. A similar reaction has been observed in several normal men without typhus history. A minimum of 50 to 100 *Rickettsiae* per oil immersion field seem to be necessary to produce the reaction. The heating of the formalinized *Rickettsiae* at 70°C. for 30 minutes renders the suspensions inactive when intradermally inoculated into normal men and guinea pigs.

The results of the intradermal inoculation into the few immune persons available in Boston have shown that heated or unheated formalinized *Rickettsiae* produced an allergic reaction in one immune person who had Mexican typhus, acquired in this laboratory in 1929; another with a similar history showed a slight reaction to the unheated *Rickettsiae*. However a few individuals tested with unheated formalinized *Rickettsiae* in the Beth Israel Hospital showed no reaction at all.

The reactions observed in normal persons and guinea pigs are probably due to the toxicity of the *Rickettsia* bodies. The toxic substances remain active in the formalinized suspensions at least 1 month, but are easily destroyed by heat.

Further studies of a combination of the allergic and toxic reactions may be of advantage in the detection of typhus immune persons.

SUMMARY AND CONCLUSIONS

The intradermal inoculation of Mexican typhus virus into immune guinea pigs produces a local reaction which is similar in its appearance to the lesion observed in the skin of normal animals submitted to the same treatment. The reaction in the immune animal appears earlier and fades sooner than the lesion in the normal guinea pig.

The inoculation of heat-killed or formalin-killed *Rickettsiae* produces no significant reactions at the site of the intradermal injection in typhus immune guinea pigs.

The virus, inoculated intradermally, has been recovered from the local lesion 72 hours after the injection into typhus immune guinea pigs.

Normal guinea pigs and persons without a history of typhus fever present a congestion and some swelling of the skin at the site of the intradermal injection of formalinized Mexican *Rickettsiae*. The reaction appears 24 hours after the inoculation and fades within 48 hours. Heating the formalinized *Rickettsia* suspensions at 70°C. for 30 minutes renders them inactive in normal men and guinea pigs.

From the experiments reported in this paper it seems that the reactions observed in typhus immune guinea pigs submitted to a

second inoculation of typhus virus, belong to the group of reactions presented by tuberculous animals (Koch's phenomenon) and the accelerated takes shown by immune persons submitted to revaccination with vaccinia virus.

A heat labile substance has been demonstrated in the formalinized *Rickettsia* bodies, which produces a reaction in the skin of normal men and guinea pigs.

STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

III. ANAPHYLAXIS INDUCED BY ARSPHENAMINE

By K. LANDSTEINER, M.D., AND JOHN JACOBS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Reference has been made in a previous paper (1) to the work of several authors (Swift, Frei, Sulzberger, Mu) on sensitization to arspenamine and neoarsphenamine. In most of the communications skin lesions or "flare ups" at the site of old injections were described. Of 19 animals given intraperitoneal or intravenous injections of neutralized arspenamine mixed with guinea pig serum Swift (2) observed, on reinjection of a similar mixture, "symptoms like those seen in anaphylaxis;" one animal succumbed in 10 minutes, and two others after some hours. Arspenamine alone, without serum, did not seem to sensitize. Kolle and Rothermund (3) after unsuccessful attempts obtained positive results by sensitizing with very small quantities of neosalvarsan and observed acute death with symptoms which had "a certain similarity to anaphylactic shock" (translated). These experiments, briefly mentioned in a discussion on allergy, have not, to our knowledge, been published in detail.

Lauf (4) injected neosalvarsan solutions without serum subcutaneously and upon intravenous reinjection observed severe anaphylactic symptoms in perhaps 15 per cent of the animals and in others slight symptoms, but in no instance did death occur in anaphylactic shock. The most striking results were obtained after a rest period of 6 months.

Working with the guinea pig uterus (Schulz-Dale method) Kallós and Kallós-Deffner (5) found that reactions occurred in 7 of 11 cases after previous subcutaneous treatment with horse serum-arsphenamine mixtures. For the tests, also, serum mixtures were used. Anaphylactic experiments other than those with the Schulz-Dale method are not mentioned. According to the authors, sensitization could be transferred passively.

Birnbaum (1934), who gives a comprehensive review of the literature (6), concludes that there is no proof for the assumption that skin manifestations in human beings following administration of arspenamine are true allergic effects, and casts doubt on the reports concerning passive transfer of arspenamine hypersensitiveness. With regard to animal experiments he remarks that "it has never

been possible, even with large doses of arsphenamine, . . . to produce anaphylactic shock" (translated).

On account of the irregularity of effects, as recorded in the literature, and the differences between results of various investigators, attempts directed toward improving the technique were thought to be desirable.

We found that, with a suitable method, intense local hypersensitive-

TABLE I*

Sensitization effects in two batches of guinea pigs similarly treated with one intracutaneous injection of 0.15 mg. arsphenamine, not neutralized, and neoarsphenamine, respectively, in 0.1 cc. saline; tested after an interval of 1 month with the respective substances.

No.	Animals sensitized to and reinjected with arsphenamine	No.	Controls
1	22, p.p.-p., el., necr.c. 4	7	6, p.p., el.
2	12, p., el., necr.c. 3	8	6, p.p., el.
3	9, p., el., necr.c. 3	9	7, f.p., sl.el.
4	26, p., swol., necr.c. 3	10	5, p.p., el.
5	18, p., el., necr.c. 3	11	5, f.p., el.
6	19, p.p., el., necr.c. 3	12	5, f.p., el.
No.	Animals sensitized to and reinjected with neoarsphenamine	No.	Controls
13	5, p.p., sl.el.	19	6, f.p., el.
14	5, f.p., sl.el.	20	6, a.cls., fl.
15	4, a.cls., sl.el.	21	5, f.p., el.
16	a.neg.	22	4, f.p., sl.el.
17	5, f.p., el.	23	6, p.p., sl.el.
18	6, p., el.	24	6, p.p., sl.el.

* The following abbreviations are used: almost colorless (a.cls.), faintly pink (f.p.), pale pink (p.p.), pink (p.). Other designations are: almost negative (a.neg.), flat (fl.), slightly elevated (sl.el.), elevated (el.), necrotic center (necr.c.).

The figures give diameters of the lesions in millimeters.

ness could regularly be obtained (1). With arsphenamine solutions, not neutralized, practically all sensitized animals gave conspicuous lesions consisting of rather large pink elevated areas almost regularly with central necrosis. When compared with effects produced by neoarsphenamine, it appeared that arsphenamine had a distinctly greater sensitizing capacity. This is evidenced by the experiment given in Table I.

Strongly positive skin reactions to arsphenamine were observed in seven experiments with more than 50 animals which in part are shown in Table II. On the first injection only faint to pale pink lesions were produced, with a diameter of 4–7 mm.

These observations prompted attempts to produce anaphylaxis in animals sensitized by this method. Preliminary experiments indicated that several injections extending over a rather long period were effective; the technique developed was as follows.

Guinea pigs were given 2 intracutaneous injections of 0.15 mg. arsphenamine, not neutralized, in 0.1 cc. saline a month apart and 3 weeks later injected intravenously with a solution of 10 mg. arsphenamine in 0.1 cc. saline and 0.09 cc. $N/1$ NaOH, made up to 1.0 cc. with normal guinea pig serum. In those animals which did not succumb to the first intravenous injection the dose was repeated after 3 weeks.

The results obtained with three batches of guinea pigs treated with the same brand of arsphenamine and by the same method, are given in Table II.

About half of the animals died in typical anaphylaxis on either the first or second injection, while still others presented distinct symptoms. It may be of interest to note that the number of guinea pigs with no or very slight symptoms, or those which died in shock, was greater than the number of animals showing symptoms of medium degree.

Of 50 controls injected intravenously in the same way as the animals previously treated, 35 showed no symptoms at all, 13 gave coughs, and 2 were slightly sick. The following drops in temperature of $0.5^{\circ}\text{C}.$ or more were shown by one animal each: -0.5° , -0.6° , -0.7° , -1.2° , and -1.4° .

When, in another batch of sensitized animals, instead of a guinea pig serum-arsphenamine mixture 5 mg. of arsphenamine, not neutralized, was injected intravenously again some of the animals succumbed with anaphylactic symptoms, seeming to indicate that serum admixture is not necessary for the success of the experiment (Table III). Whether it offers a distinct advantage remains to be investigated in more extensive experiments.

In all, of 56 treated guinea pigs, 30 died in typical anaphylaxis, and a number of others showed distinct symptoms, whereas, as already

indicated, none of the 50 controls injected in the same manner as the treated guinea pigs died or developed severe symptoms.

TABLE II

Combined table. Sensitization with arsphenamine (Winthrop Chemical Company). The second column gives the diameter of the skin lesions in millimeters. Figures in parentheses indicate change in temperature ($^{\circ}\text{C}.$).

No.	Second intracutaneous injection	First intravenous injection	Second intravenous injection
		Symptoms	Symptoms
	<i>mm.</i>		
25	22	None (-0.1)	None (0)
26	22	" (-0.9)	" (-0.5)
27	14	Slight (-1.9)	† 7 min.
28	17	" (-1.0)	† 8 "
29	23	† 19 min.	
30	18	Slight (-1.1)	Slight (-0.5)
31	16	None (-0.4)	† 8 min.
32	7	" (0)	Moderate ($+0.2$)
33	14	Slight (-2.7)	Severe (-3.7)
34	21	" (-1.8)	None
35	20	† 6 min.	
36	22	† 3 "	
37	10	Moderate (-1.6)	Slight (0)
38	25	† 20 min.	
39	21	Moderate (-1.0)	None ($+0.2$)
40	15	† 5 min.	
41	25	† 7 "	
42	9	† 4 "	
43	17	Slight (0)	† Overnight
44	27	† 38 min.	
45	7	None ($+0.1$)	None (0)
46	18	" (0)	" (0)
47	17	† 19 min.	
48	21	None (0)	None ($+0.2$)
49	20	† 4 min.	

† Designates death. Animals dying in a short time all showed the typical picture (lungs inflated, heart beating) on autopsy.

In a similar experiment with 5 animals an arsphenamine preparation, procured from the Abbott Laboratories, was used. In this lot 2 animals died on the second intravenous injection.

A point of special significance is that in the experiments reported a synthetic chemical substance, without being used in chemical combination with protein, produces skin sensitization as well as the anaphy-

lactic state. It will be of interest to follow up the mechanism of this sensitization.

TABLE III

Sensitization effects in a batch of guinea pigs treated with two skin injections a month apart of 0.15 mg. arsphenamine, not neutralized, (Winthrop Chemical Company) and 3 weeks after the second injection, injected intravenously with 5.0 mg. of the same preparation in 1.0 cc. saline. The second, similar intravenous injection was given 3 weeks after the first. Figures in parentheses indicate change in temperature ($^{\circ}\text{C}.$).

No.	First intravenous injection	No.	Second intravenous injection
	Symptoms		Symptoms
50	Slight (-3.5)		None (0)
51	None (0)		Moderate (-1.0)
52	" (-0.1)		Coughs (0)
53	† 8 min.		
54	Slight (-2.5)		† 16 min.
55	Moderate (-4.4)		† 51 "
56	None (0)		Coughs ($+0.1$)
	Controls		Controls
57	None (-0.1)	64	None (0)
58	" ($+0.1$)	65	" ($+0.2$)
59	" ($+0.2$)	66	" ($+0.1$)
60	" ($+0.1$)	67	" (0)
61	" (-0.1)	68	" (0)
62	" (0)	69	" ($+0.1$)
63	" (0)	70	" (0)

SUMMARY

Experiments are described which show that with a given treatment guinea pigs can be sensitized to arsphenamine, so that a considerable percentage die in anaphylactic shock on intravenous administration of the substance.

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AN ACQUIRED RESISTANCE OF GROWING ANIMALS TO CERTAIN NEUROTROPIC VIRUSES IN THE ABSENCE OF HUMORAL ANTIBODIES OR PREVIOUS EXPOSURE TO INFECTION*

BY PETER K. OLITSKY, M.D., ALBERT B. SABIN, M.D., AND
HERALD R. COX, Sc.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The widespread immunization of a susceptible, growing population as a result of exposure to various infectious agents is well known. The purpose of this communication, however, is to present evidence that certain animals may with increasing age acquire resistance to peripheral inoculation with some neurotropic viruses without any previous exposure to infection and in the absence of demonstrable humoral antibodies.

In the course of experiments with the virus of vesicular stomatitis in mice, certain irregularities in susceptibility were observed when the virus was injected by routes other than the intracerebral, and it was conjectured that some of the variations might be due to a lack of uniformity in age among the animals used. A series of experiments was then undertaken to test quantitatively the incidence of morbidity and death among mice of known age following inoculation of this virus by various routes. The study was subsequently extended to include similar tests with other viruses.

Animals and Methods

Unless designated otherwise the mice used in this investigation are generally referred to as The Rockefeller Institute albino strain. While they do not represent a pure genetic strain they have been inbred at this Institute for about 24 years. Mice of known age were therefore readily available. Their weight was not a reliable criterion by itself, since it varied with the number of mice in a litter and

* Preliminary report given at meeting of American Association of Pathologists and Bacteriologists, April, 1935; for abstract see *Am. J. Path.*, 1935, 11, 839.

other conditions. These mice had no opportunity for exposure to the viruses under investigation until used. Intracerebral injections were given under ether anesthesia, while intranasal and other peripheral inoculations were made without anesthesia. Suspensions of virus were usually prepared from fresh tissue in broth, unless otherwise stated. The supernatant liquid of a 10 per cent suspension, after horizontal centrifugation at about 2000 R.P.M. for 10 minutes, was taken to represent a virus dilution of 1:10, from which further tenfold dilutions were prepared in broth, always using a sterile pipette for each new dilution.

Experiments with Vesicular Stomatitis Virus (Indiana and New Jersey Strains)

Both the Indiana and New Jersey strains of vesicular stomatitis virus which are immunologically distinct, have undergone numerous brain to brain passages in mice (1). Although in nature, in horses and cattle, this virus has not thus far been observed to induce encephalitis (2-4), it has done so after experimental intracerebral inoculation in monkeys, rabbits, guinea pigs, rats, and mice and after intranasal and others ways of peripheral inoculation in mice (5).

Susceptibility of Young and Old Mice to Infection by Intracerebral and Intranasal Routes.—In order to accentuate any possible variations which might be attributable to differences in age, mice at extremes of age were employed. The young ones were 14 days of age and their average weight was 7.2 gm.; the other group consisted of animals about 1 year old and of an average weight of 35.4 gm. Dilutions of virus ranging from 10^{-4} to 10^{-8} were injected intracerebrally and from 10^{-1} to 10^{-8} by way of the nose; 0.03 cc. was the amount given by both methods, 3 mice being used for each dilution and route.

The results, shown in Table I, indicate no appreciable difference in the incidence of morbidity and of death between the young and old mice when the Indiana strain of the virus was injected directly in the brain, while a distinct difference in the two age groups was evident after intranasal instillation. In the intracerebral group, the old mice generally showed signs of disease and a lethal outcome after a longer incubation period, but the titration end-point was practically the same as for the young mice. After nasal instillation of the virus, on the other hand, none of the old mice developed encephalitis or exhibited any other signs of disease, while in the young mice the 10^{-4} dilution of virus was fatal for all, and 10^{-5} for one of three.

The same type of experiment was performed with the New Jersey strain of the virus, and the results, given in Table II, are practically the same as those with the Indiana strain. All but one of the old mice, receiving the virus by way of the nose, remained well; the exceptional one showed signs of encephalomyelitis on the 8th day and survived with residual paralysis of the posterior extremities. Two old mice were given 0.1 cc. each of the 1:100 virus dilution intranasally instead of 0.03 cc., to determine whether the larger volume might influence the

TABLE I

Susceptibility of Young and Old Mice to Intracerebral and Intranasal Inoculations with Vesicular Stomatitis Virus (Indiana Strain)

Route of inoculation	Dilution of virus	Age of mice and result	
		14 days old, average weight, 7.2 gm.	About 1 year old, average weight, 35.4 gm.
Intracerebral	10^{-4}	n. t.*	3, 3, 4†
	10^{-5}	n. t.	4, 4, 4
	10^{-6}	2, 3, 0	5, 5, 7
	10^{-7}	3, 0, 0	0, 0, 0
	10^{-8}	0, 0, 0	0, 0, 0
Intranasal	10^{-2}	n. t.	0, 0, 0
	10^{-3}	n. t.	0, 0, 0
	10^{-4}	5, 7, 7	0, 0, 0
	10^{-5}	7, 0, 0	0, 0, 0
	10^{-6}	0, 0, 0	0, 0, 0
	10^{-7}	0, 0, 0	0, 0, 0
	10^{-8}	0, 0, 0	0, 0, 0

* n. t. = not tested.

† Numbers indicate the day of death of individual mice, 0 signifying survival.

outcome, but both remained well. Numerous other old and young mice have since received nasal instillations of vesicular stomatitis virus and the old ones with occasional exceptions were resistant while the young ones invariably succumbed to encephalitis.

The fact that there is no appreciable age difference when the virus is injected directly into the brain suggests that the portions of the central nervous system, involvement of which leads to clinical encephalitis and death, are apparently fully susceptible to the action of the

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virus, and that the resistance to infection by way of the nose encountered in old mice is not a general or systemic one. It would appear rather that some as yet unknown variation in the pathogenesis of the disease or the spread of the virus from the periphery might account for the resistance of the older animals.

Susceptibility of Young and Old Mice to Inoculation by Other Peripheral Routes.—To determine whether the resistance of the older animals was due to some unique condition present in the nasal path or merely to the result of peripheral inoculation as such, young and

TABLE II
Susceptibility of Young and Old Mice to Intracerebral and Intranasal Inoculation with Vesicular Stomatitis Virus (New Jersey Strain)

Route of inoculation	Dilution of virus	Age of mice and result	
		15 days old, average weight, 8.2 gm.	About 1 year old, average weight, 35.8 gm.
Intracerebral	10^{-6}	2, 2, 0	3, 3, 4
	10^{-6}	2, 2, 4	4, 5, 0
	10^{-7}	0, 0, 0	4, 0, 0
Intranasal	10^{-1}	n. t.	0, 0, 0
	10^{-2} (0.03 cc.)	n. t.	0, 0, p. p. 8*
	10^{-2} (0.1 cc.)	n. t.	0, 0
	10^{-3}	7, 7, 11	0, 0, 0
	10^{-4}	7, 11, 0	n. t.
	10^{-5}	0, 0, 0	n. t.

Abbreviations as in Table I.

*p. p. 8 = paralysis of posterior extremities 8 days after inoculation, but survived.

old mice were injected with varying dilutions of vesicular stomatitis virus (New Jersey strain) subcutaneously, intramuscularly, intraperitoneally, and intravenously.

The subcutaneous inoculations were made in the back of the lower thoracic and lumbar regions, 0.5 cc. of the various dilutions of virus (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) being injected into each of three young and three old mice. None of the old mice exhibited any signs of disease and all survived, while the young mice developed first flaccid paralysis of the posterior extremities; the paralysis ascended and the animals died within 24 to 48 hrs. All three mice injected with the 10^{-1} dilution

of virus, two of three with 10^{-2} and 10^{-3} , and one of three with 10^{-4} dilution were affected (Table III).

The *intramuscular inoculations* were given into the calf muscles of one side. Six young and six old mice were each injected with 0.3 cc. of the 10^{-1} dilution (*i.e.* 10 per cent centrifuged suspension). The old mice remained well while the young ones developed flaccid paralysis of the posterior extremities (the inoculated extremities frequently exhibiting paralysis first) and later died (Table III).

Certain difficulties were encountered with the *intravenous inoculations* because of the well known immediate toxicity of homologous brain suspensions and the small size of the tail veins in very young mice. However, four 1 year old animals which survived an injection of 0.2 cc. of 1:20 centrifuged suspension of virus

TABLE III

Susceptibility of Young and Old Mice to Inoculation by Various Peripheral Routes with Vesicular Stomatitis Virus (New Jersey Strain)

Route and amount of inoculation	Dilution of virus	Age of mice and result	
		15 days old, average weight, 10 gm.	About 1 year old, average weight, 35 gm.
Subcutaneous, 0.5 cc. each	10^{-1}	7, 8, 12	0, 0, 0
	10^{-2}	7, 8, 0	0, 0, 0
	10^{-3}	7, 7, 0	0, 0, 0
	10^{-4}	12, 0, 0	0, 0, 0
Intraperitoneal, 0.5 cc. each	10^{-1}	0, 0, 0	0, 0, 0
	10^{-2}	0, 0, 0	0, 0, 0
	10^{-3}	0, 0, 0	n. t.
	10^{-4}	0, 0, 0	n. t.
Intramuscular, 0.3 cc.	10^{-1}	4, 4, 5, 5, 5, 6	0, 0, 0, 0, 0, 0

Abbreviations as in Table I.

remained entirely well. Eight 21 day and two 16 day old mice were injected with 0.2 cc. of a 1:200 dilution, but with the exception of two 21 day mice, a certain amount escaped in the tissues of the tail. Four of the eight 21 day mice (including only one of the two in which the injection appeared to be entirely intravenous) and both 16 day mice developed flaccid paralysis of the posterior extremities within 5 to 6 days and died 24 hours later. The development of flaccid paralysis of the posterior extremities as the first nervous manifestation after intravenous inoculation is highly suggestive of a centripetal spread of the virus by way of the nerves supplying the abdominal viscera.

Intraperitoneal inoculations of 0.5 cc. of various virus dilutions were made in 15 day and 1 year old mice, and as noted in Table III, neither the young nor the

TABLE V
Age When Mice Become Resistant to Nasal Instillation of Vesicular Stomatitis Virus

Date of experiment and strain of virus	Strain of mice	Dilution of virus and No. of i. c. M.I.D.*	Age	Average weight	Result
Feb. 21, 1935, Indiana	Rockefeller Institute albino	1:100 (10,000)	days	gm.	
			11	5.3	5, 5, 6
			20	9.5	7, 7, 7
			31	15.8	9, 0, 0
			56	24.5	9, p. p. 14, 0
Mar. 20, 1936, New Jersey	Rockefeller Institute albino	1:100 (10,000)	About 360	37.6	14, 0, 0
			14	5.1	5, 5, 5, 5, 6, 6
			21	6.8	5, 5, 5, 5, 6, 6
			30	11.8	6, 6, 7, 7, 8, 0
			48	24.5	③, † 7, 9, 11, 11, 0
Mar. 31, 1936, New Jersey	Rockefeller Institute albino	1:100 (1000-10,000)	About 360	38.7	9, p. p. 10, p. p. 10, 0, 0, 0
			18	7.6	5, 6, 7, 0, 0, 0
			240-360	38.8	0, 0, 0, 0, 0, 0
			18	7.6	5, 5, 6, 6, 0, 0
			240-360	38.8	0, 0, 0, 0, 0, 0
	Webster virus-susceptible	1:100 (1000-10,000)	18	7.1	5, 6, 0
			90	35.2	②, ③, 0
			Over 180	41.0	0, 0, 0
			18	7.1	8, 0, 0
			90	35.2	0, 0, 0
	Webster virus-resistant	1:100 (1000-10,000)	Over 180	41.0	0, 0, 0
			19	8.3	5, 7, 0
			90	28.1	0, 0, 0
			About 360	36.0	0, 0, 0
			19	8.3	5, 7, 7
June 6, 1936, New Jersey	Webster virus-resistant	1:100 (10,000-100,000)	90	28.1	0, 0, 0
			About 360	36.0	0, 0, 0
			31	14.8	7, 8, 9, 9, 10, 0, 0
	Rockefeller Institute albino	1:100 (10,000-100,000)	15	8.6	4, 4, 4, 4, 5, 5
			31	16.9	5, 6, 6, 7, 8, 0
			About 360	35.5	11, 0, 0, 0

Abbreviations as in previous tables.

*i.c. M.I.D. = minimal infective doses by intracerebral route.

† ③ signifies that death was not caused by the virus.

Half the number of mice of each age and group received a 1:100 glycerolated virus suspension and the other half a 1:1000 dilution. The results (Table V) indicate no significant difference in susceptibility among the mice of the three groups. With the 1:100 dilution of virus in the 18 or 19 day old mice, three of the six Institute animals, two of three virus-susceptible and two of three virus-resistant mice succumbed, while with the 1:1000 virus dilution, four of six Institute mice, one of three virus-susceptible, and all three virus-resistant ones developed the virus encephalitis. None of the twelve 8 month to 1 year old Institute mice developed the disease, nor did any of the twenty-four virus-susceptible or virus-resistant animals 90 days or more of age, succumb to virus encephalitis. In the last experiment recorded in Table V, 31 day old mice from the Institute albino and Webster virus-resistant stocks were compared. It may be noteworthy that the seven virus-resistant mice in this test constituted a single litter from the mating of 3 month old virus-resistant parents used in the previous experiment. There is again no appreciable difference between the number of 31 day old virus-resistant and ordinary Institute mice that succumbed to nasal infection, although the incubation period appeared to be rather longer in the former.

It should be noted here that while the mice designated as relatively virus-susceptible and virus-resistant have been shown to exhibit these variations in susceptibility as regards the viruses of louping ill and St. Louis encephalitis (6, 7), it is evident that this does not obtain for the virus of vesicular stomatitis (New Jersey strain), and as recently observed by Webster (8) also not for the virus of rabies. From the point of view of the special type of resistance only to peripheral inoculation, described in this communication, there is thus no evidence as yet for considering its development with increasing age as genetically predetermined. Further studies in the direction of the effects of genetic, dietetic, and physiologic factors are, however, indicated.

Experiments with Other Neurotropic Viruses²

The influence of the age of different hosts on the infectivity of other neurotropic viruses, inoculated intracerebrally and peripherally, will now be described.

² The term neurotropic refers to the capacity of a virus to attack nervous tissue but does not imply that it may not also affect other tissues.

Eastern Equine Encephalomyelitis Virus in Mice.—Mice 15 days and 1 year of age were given various dilutions of Eastern equine encephalomyelitis virus (mouse brain suspension in broth) by the intracerebral and intranasal routes as indicated in Table VI. It may be seen that no appreciable difference in susceptibility exists between the young and old mice inoculated intracerebrally, while only a slight variation is evident in the nasally infected ones. After nasal instillation the old mice succumbed on the average a day or two later than the young, and in the latter the virus was apparently infective in a tenfold

TABLE VI
Susceptibility of Young and Old Mice to Intracerebral and Intranasal Inoculations of Eastern Equine Encephalomyelitis Virus

Route of inoculation	Dilution of virus	Age of mice and result	
		15 days old, average weight, 10 gm.	About 1 year old, average weight, 30.5 gm.
Intracerebral	10^{-6}	2, 2, 2	3, 3, 3
	10^{-7}	3, 3, 0	3, 0, 0
	10^{-8}	3, 3, 3	3, 6, 0
Intranasal	10^{-1}	n. t.	4, 4, 4
	10^{-2}	3, 3, 4	4, 5, 0
	10^{-3}	3, 4, 4	5, 5, 6
	10^{-4}	4, 4, 4	5, 0, 0
	10^{-5}	9, 0, 0	n. t.
	10^{-6}	0, 0, 0	n. t.

Abbreviations as in previous tables.

greater dilution. Whether or not a different pathogenesis in the case of Eastern equine encephalomyelitis is the responsible factor, it is manifest that its infectiousness is influenced by the age of the host neither in the same manner nor to the same degree as is that of the two strains of vesicular stomatitis virus.

Western Equine Encephalomyelitis in Mice.—Two sets of titrations were carried out with this virus (Table VII). In the first 21 day and 1 year old mice and in the second, 15 day and 1 year old animals were injected intracerebrally and instilled intranasally. It is evident that after intracerebral inoculation there was little or no difference in

suceptibility between the 21 day and 1 year old mice, while the 15 day animals succumbed more regularly than the old ones; in these death ensued in half the time even when a minimal dose was given. After intranasal inoculation there was a distinct quantitative difference in effect in young and old mice, which was not as marked, however, as that observed with the viruses of vesicular stomatitis. Of eighteen 15 or 21 day old mice instilled with virus in dilutions of 1:10 to

TABLE VII

Susceptibility of Mice of Various Ages to Western Equine Encephalomyelitis Virus by Intranasal and Intracerebral Routes

Route of inoculation	Dilution of virus	Experiment I		Experiment II	
		21 days old, average weight, 14.1 gm.	About 1 year old, average weight, 32 gm.	15 days old, average weight, 8 gm.	About 1 year old, average weight, 35 gm.
Intracerebral	10^{-1}	3, 4, 4	3, 4, 4	n. t.	n. t.
	10^{-2}	3, 3, 4	3, 4, 4	"	"
	10^{-3}	3, 4, 4	3, 4, 4	"	"
	10^{-4}	3, 3, 5	3, 3, 4	2, 2, 2	3, 3, 4
	10^{-5}	3, 3, 4	3, 4, 4	2, 2, 2	4, 4, 10
	10^{-6}	3, 3, 4	4, 4, 5	2, 2, 2	4, 4, 0
	10^{-7}	3, 4, 5	4, 0, 0	2, 2, 2	4, 4, 0
	10^{-8}	n. t.	n. t.	2, 2, 2	6, 0, 0
Intranasal	10^{-1}	4, 7, 7	5, 5, 0	n. t.	n. t.
	10^{-2}	3, 3, 4	4, 0, 0	3, 3, 3	5, 7, 0
	10^{-3}	3, 3, 4	5, 0, 0	2, 2, 6	5, 5, 0
	10^{-4}	4, 4, 4	0, 0, 0	3, 3, 3	0, 0, 0
	10^{-5}	0, 0, 0	0, 0, 0	4, 0, 0	0, 0, 0
	10^{-6}	0, 0, 0	0, 0, 0	3, 4, 0	0, 0, 0
	10^{-7}	0, 0, 0	0, 0, 0	n. t.	n. t.

Abbreviations as in previous tables.

1:10,000, all died of encephalomyelitis, while of a similar number of 1 year old animals inoculated with the same material only eight succumbed. Furthermore while three of six 15 day old mice died after nasal instillation with virus dilutions of 10^{-5} and 10^{-6} , none of the old ones was affected by these or larger amounts (10^{-4} dilution) of the infective material.

Pseudorabies in Guinea Pigs.—10 day old guinea pigs having an

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average weight of 140 gm. and full grown guinea pigs, 4 months of age or older and of an average weight of 660 gm., were given subcutaneous

TABLE VIII
Susceptibility of Young and Old Guinea Pigs to Intranasal and Subcutaneous Inoculations of Pseudorabies Virus

Route of inoculation and amount injected	Dilution of virus	Age of guinea pigs and result	
		10 days old, average weight, 140 gm.	4 months old, average weight, 660 gm.
Intranasal, 0.05 cc. in each nostril	10 ⁻¹	3, 3	3, 3
	10 ⁻²	3, 0	3, 0
	10 ⁻³	0, 0	0, 0
	10 ⁻⁴	0, 0	0, 0
Subcutaneous, 1 cc.	10 ⁻¹	3, 3	3, 3
	10 ⁻²	3, 3	3, 0
	10 ⁻³	3, 0	4, 0
	10 ⁻⁴	0, 0	0, 0

Abbreviations as in previous tables.

TABLE IX
Relative Susceptibility of Young and Old Monkeys to Nasal Instillation of Polio-myelitis Virus

Average weight of monkeys tested	Monkey No.	Result
Weight, 1 kilo, (about $\frac{1}{3}$ the size of monkeys ordinarily used)	1	Remained well
	2	Fever 20, par. 23, prostr. 25*
	3	Fever 5, par. 8, postr. 10
	4	Fever 6, par. 10, postr. 14
Age unknown but have been at Institute 3 to 5 years. Average weight, 7 to 8 kilos	5	Fever 7, par. 11, prostr. 12
	6	Fever 7, par. 10, prostr. 11
	7	Fever 6, par. 10, prostr. 15
	8	Fever 7, par. 11, prostr. 13
	9	Fever 4, par. 8, prostr. 9

* Fever 20, par. 23, prostr. 25 = Fever 20th day, paralysis 23rd day, prostrate 25th day after the first instillation of virus.

ously and intranasally various dilutions of the same virus suspension (glycerolated rabbit brain). The results (Table VIII) reveal no differ-

ence in susceptibility of young and old guinea pigs to peripheral inoculation with pseudorabies virus.

Poliomyelitis in Macacus rhesus Monkeys.—It is difficult (and for us it proved impossible) to obtain monkeys of known age. The *rhesus* monkeys usually employed for experimental work weigh 2 to 3 kilos; to induce the disease regularly by means of nasal instillation of virus a relatively large amount of it is given. It was therefore desirable to test with the same dose the susceptibility of the smallest and largest monkeys available. Four very small monkeys (average weight 1 kilo) and five large, mature animals (average weight 8 kilos), which have been kept at the laboratory for 3 to 5 years, were given the same quantity of poliomyelitis virus (1 cc. of 10 per cent virus suspension in each nostril on two occasions, 48 hours apart). As may be seen in Table IX, all of the old monkeys succumbed while only three of the four young ones developed poliomyelitis. These results are in striking contrast to the statement made (9) that old *Macacus rhesus* monkeys are less susceptible to experimental poliomyelitis. In view of the fact that neurotropic viruses may behave differently in different hosts, one cannot assume that poliomyelitis virus would necessarily act in a similar manner in older animals of other species.

DISCUSSION

The opinion is often expressed that in general young animals are more susceptible than old ones to virus inoculations, but no precise comparative study of this question has heretofore been made. It has been recorded, for example, that young rabbits succumb more rapidly after intracerebral injection of Borna disease virus than older animals (Nicolau and Galloway (10)); that young monkeys are more susceptible than old ones to intracerebral inoculation with poliomyelitis virus (Leiner and von Wiesner; Krause and Meinicke; Levaditi; and Jungeblut and Engle) (9); that yellow fever (Theiler (11)) and vesicular stomatitis viruses (Olitsky, Cox, and Syverton (5)) are encephalitogenic after intraperitoneal inoculation only in very young mice, etc. It should be recalled here that the resistance of older animals to many other virus diseases is chiefly the result of infection at an earlier age.

The present investigation provides evidence of a different and rather

more limited type of resistance which develops in some animals with increasing age only as regards certain neurotropic viruses introduced into the body by definite peripheral routes. This type of resistance is not general and systemic in nature, for introduction of the virus directly into the brain finds both young and old equally susceptible. Previous exposure to infection is eliminated as a possible factor not only by the isolation of the animals in the breeding rooms prior to their use but also by the complete lack of antiviral bodies in the blood of those naturally resistant.

This naturally acquired resistance was found to be most marked in old mice against the Indiana and New Jersey strains of vesicular stomatitis virus. It was demonstrable when inoculations were made by the intranasal, subcutaneous, intramuscular, intraperitoneal, and intravenous routes, but not by the intracerebral. The development of this resistance varied in different mice, becoming apparent as early as the 30th day of life in some and remaining absent in others even at 1 year of age. The factors which influence the development of the refractory state and its nature are still obscure. It should be stressed that the change which renders old mice refractory to peripheral inoculation with the two immunologically distinct strains of vesicular stomatitis virus does not necessarily induce a similar state against other viruses. Thus, while the majority of older mice exhibit a similar resistance against the Western strain of equine encephalomyelitis virus, it is not readily demonstrable with the immunologically distinct Eastern strain. Nor were older animals refractory to peripheral inoculations of pseudorabies virus in guinea pigs and poliomyelitis virus in *rhesus* monkeys. The mechanism of the acquired resistance described in this communication has been studied, and a preliminary report published (Sabin and Olitsky (12)).

CONCLUSIONS

1. As mice grow older they acquire a resistance to peripheral inoculation with the Indiana and New Jersey strains of vesicular stomatitis virus and to some extent also to Western equine encephalomyelitis virus, but little or none to the Eastern strain.
2. While some mice may become resistant as early as the 30th day of life, others may still be susceptible at 1 year of age.

3. This resistance is readily demonstrable when the inoculations are made by intranasal, subcutaneous, intramuscular, intraperitoneal, and intravenous routes, but not when the virus is injected directly into the brain.

4. The resistance is not related to previous exposure to infection or to the presence of specific or nonspecific antiviral bodies in the blood.

5. No difference in susceptibility to peripheral inoculation was found in young and old guinea pigs to pseudorabies virus, and in relatively young and old *Macacus rhesus* monkeys to poliomyelitis virus.

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HUMORAL ANTIBODIES AND RESISTANCE OF VACCINATED AND CONVALESCENT MONKEYS TO POLIOMYELITIS VIRUS

By ALBERT B. SABIN, M.D., AND PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The observation has been made frequently (1-5) that monkeys actively immunized with different preparations of the virus of poliomyelitis while developing humoral antibodies, often do not resist either intracerebral or intranasal inoculations of the virus. In this paper we shall present our experiences with the nasal instillation in monkeys which have passed through clinically perceptible attacks of experimental poliomyelitis and the correlation of the effects produced with humoral antibodies present as quantitatively ascertained. The study has practical significance in determining whether a certain concentration of antiviral bodies in vaccinated monkeys has the same value as regards protection to infection as it has in convalescents.

The question of reinfection in convalescent monkeys has been studied by Flexner (6) in relation especially to second attacks of the disease in children. Contrary to accepted views, he has found that reinfection takes place in nasally instilled monkeys, and that the second attack may sometimes be induced by the same although apparently oftener by a foreign strain of virus; and he has also made tests for the presence of humoral antibodies to both kinds of strains in the reinfected animals (personal communication).

EXPERIMENTAL

Reaction of Convalescent Monkeys to Nasal Instillation of Virus.—As early as 1910, Flexner (7) and later others (8a, 9) showed that monkeys convalescent from experimental poliomyelitis are, with only rare exceptions, resistant to intracerebral inoculation of the same strain of virus.

Monkeys inoculated with the Rockefeller Institute strain of mixed virus (M. V.) which was used in this study rarely recover after the development of paralysis; the collection of a suitable number of convalescents for this investigation was therefore no easy task (*cf.* Flexner (7, 10)). Of the nine monkeys studied, five were originally infected with virus by way of the nose and four by intracerebral injection. The intranasal test for susceptibility consisted of two instillations of virus, 48 hours apart. A 10 per cent suspension in saline was used of a mixture of glycerolated cords from at least four monkeys, paralyzed after nasal infection, and 1 cc. was instilled in each nostril. At least three or four normal monkeys received the same virus suspension by the same route, whenever any of the convalescents were being tested. During this study thirty control monkeys were used and all developed poliomyelitis.

Of the nine convalescent monkeys given one or more series of instillations at monthly intervals, six resisted the intranasal tests and three died in a peculiar manner.

Flexner (11) has shown that the bringing of virus into contact with the nasal membrane is never an indifferent process in monkeys and that both normal and convalescent animals respond to its presence with changes in the cerebrospinal fluid consisting of mononuclear pleocytosis and even of globulin; and this response takes place largely independently of the appearance of obvious clinical symptoms of disease. Certain monkeys are highly resistant to the nasal instillation of virus, but these exceptional animals still react with the changes in the cerebrospinal fluid, from which Flexner concluded that "the refractory state, therefore, resides apparently in the nerve cells, the principal seat of usual virus attack—not in the nervous tissues as a whole." The resistance of the animals in our series was measured by the complete absence of fever or other signs of disease as contrasted with the uniform occurrence of paralysis among the control monkeys in each experiment.

The histories of the three animals which succumbed in an unusual manner are as follows:

Macacus rhesus 1-82, the first of the series to succumb, was completely paralyzed after the first nasal infection. It recovered some function, however, and 3 months later was again submitted to nasal instillation of virus. 4 days after the first instillation and within less than 48 hours after the second, it was found dead; the only signs before death appeared to be increased weakness and subnormal temperature (99.2° and 96.7°F.) on the 2nd and 3rd days. The cause of death was not investigated in this case, because the rapid course did not suggest poliomyelitis.

Subsequently, however, two additional monkeys (Nos. 3-07 and 3-26) died in a similar manner after nasal instillation of virus. Monkey 3-26 developed paraly-

sis of only the lower extremities following the original intracerebral inoculation,¹ but remained otherwise well and active with normal temperature (about 102°F.) for 1 month, when it was given virus intranasally. The temperature dropped to 100.2°F. the next day, and to 100°F. on the 2nd day, when it appeared sick and seemed to breathe with difficulty; it was given still another nasal instillation of virus and was found dead the following morning. Necropsy revealed no pathological changes in the lungs or viscera. The olfactory bulbs were tested for virus, but contained none, and microscopic examination of sections of the central nervous system revealed only old poliomyelitis lesions. Monkey 3-07 exhibited complete paralysis of the left arm and some weakness of the other extremities as a result of the original intracerebral inoculation but was otherwise well and active with normal temperature (about 102.4°F.) for 5 weeks, when it was submitted to the same test as monkey 3-26. For the next 2 days it exhibited no change either in temperature or in physical condition. On the 3rd day, or 24 hours after the second nasal instillation of virus, its temperature dropped to 100.7°F.; it appeared ill and had difficulty in breathing. On the 4th day it was almost prostrate, temperature 97.6°F., and respiration exceptionally difficult and of irregular rhythm. It was anesthetized and sacrificed at this stage; the lungs and viscera showed no evidence of disease, the central nervous system showed no gross changes, and microscopically there was evidence of only old poliomyelitis lesions. The olfactory bulbs, thalamic region, pons and medulla were tested for the presence of poliomyelitis virus but none was found. It may be pointed out that another convalescent monkey, No. 4-2, was given the same virus suspension simultaneously with monkeys 3-07 and 3-26, but remained entirely well, and that three normal controls developed typical poliomyelitis after the usual course of fever and within the usual time. None of the other convalescent monkeys tested subsequently by the same procedure exhibited any abnormal signs.

From the evidence presented here one cannot attribute the deaths of these three convalescent monkeys to a second attack of poliomyelitis. Although there are reports of certain reactions in convalescent monkeys (such as a rapid rise in temperature after intracerebral inoculation (12) or rapid death after intrasplenic injection of virus (13) which have been interpreted as allergic or anaphylactic manifestations, one cannot be at all certain that such phenomena played a part in the cases just described. At the same time it is difficult to dismiss these three deaths as merely coincidental.

Neutralizing Antibodies in Convalescent Monkeys.—All the convalescent monkeys were bled at monthly intervals after the onset of paraly-

¹ All such operations were made with the aid of deep ether anesthesia.

TABLE I
Serum Antibodies and Resistance to Nasal Infection of Poliomyelitis Convalescent M. rhesus Monkeys

Monkey No.	Route of primary inoculation and extent of initial paralysis	Time since onset of disease wks.	Extent of paralysis at time of test	Antibodies in serum	Result of nasal instillation of virus*
1-82	Intranasal; paralysis of all extremities; almost prostrate	12	Marked residual paralysis but able to sit up	Not tested	Dead 4th day†
3-07	Intracerebral; complete paralysis of left arm and weakness of other extremities; up and about	3.5 5	Unchanged "	Negative " +	Not tested Prostrate with marked, irregular dyspnea—4th day. Sacrificed†
3-26	Intracerebral; paralysis only of lower extremities. Otherwise active	4	"	"	Dead 3rd day†
42	Intranasal; paralysis right arm and weakness of other extremities	4 8 12	Almost complete recovery " " "	" Positive "	Remained well " "
7-31	Intracerebral; paralysis of all extremities; almost prostrate	4 8	Improved; can sit up " gets about	Negative Positive	" Sacrificed 2nd day
7-20	Intranasal; paralysis of left upper extremity only	8 13	Almost entirely well " "	Negative Positive	Remained well " "
7-19	Intranasal; complete paralysis of lower extremities and partial paralysis of upper extremities	8 13	Sits up; improved function of upper extremities " "	" "	" "

43	Intranasal; fever and facial paralysis for few days	4 8 12	Entirely well " " " "	Not tested Negative† Positive	" " " " " "
34-15	Intracerebral; widespread partial paralysis	4 8	Almost complete recovery " " " "	Not tested Positive	Resisted intracerebral inoculation Remained well

* Controls not tabulated; see text.

† See text for complete record.

‡ Tests repeated with same result.

sis and prior to reinoculation, in order to determine whether or not they possessed demonstrable antibodies at the time they were tested for resistance to reinfection (Table I).

The neutralization test was performed in the same manner as that previously used by Olitsky and Cox (2) for demonstrating antibody in the serum of vaccinated monkeys, whose resistance to infection is now being compared with that of the convalescents. In brief, 0.2 cc. of a Berkefeld N filtrate of a 5 per cent suspension of poliomyelitis cords in saline solution was mixed with 0.8 cc. of the serum, incubated 2 hours at 37°C., and overnight in the refrigerator, and the whole mixture injected intracerebrally in a monkey. The amount of virus in this mixture represented approximately twenty minimal infective doses.

It should be noted that none of the sera obtained from five monkeys had any demonstrable antibodies 4 to 5 weeks after the onset of paralysis; at 2 months the sera of only two of six monkeys tested failed to neutralize, while at 3 months these two also exhibited antiviral bodies. Many of the neutralization tests were repeated several times with the same results; hence it is clear that the development of antiviral bodies in convalescent monkeys is generally quite slow and at times may require as long as 3 months to become demonstrable. Reports of the presence of antibody as early as 36 hours after paralysis (8b) should, therefore, be regarded either as exceptional or as the result possibly of misinterpretation of a single test. Leake (14) reported the absence of neutralizing antibodies in a monkey 1 month after the onset of poliomyelitis, and Aycock and Kramer (15) found no antibody in two convalescent sera obtained 4 to 6 weeks after paralysis, although at 6 months after the disease the sera of these animals neutralized the virus.

Antibody in the Preparalytic Stage.—In a report published after the completion of the above experiments, Jungeblut (9) stated that antibodies appear first during the preparalytic stage, disappear rapidly during the onset of paralysis, and then reappear slowly during convalescence. This conclusion is based on the observation that of the sera of nine monkeys in the preparalytic stage, four completely neutralized 0.2 cc. of a 10 per cent virus suspension, one partially neutralized (as reflected by prolongation of the incubation period for more than 14 days), and the remainder failed to neutralize. When paralysis ensued, however, the monkeys which previously had demonstrable

antibodies now showed none. In an attempt to repeat this finding, the sera of six monkeys in the preparalytic stage were tested against 0.2 cc. of 5 per cent virus filtrate (Berkefeld N), but none of them neutralized.

TABLE II

Titration of Antiviral Substance in Sera of Monkeys Which Resisted or Succumbed to Nasal Infection with Poliomyelitis Virus

Source of serum	Amount of serum added to 0.2 cc. of 5% Berkefeld N filtrate*	Result of test
	cc.	
From three convalescent monkeys which <i>resisted</i> repeated attempts at reinfection by way of nose	0.8	Neutralization
	0.4	Partial neutralization?—paralysis after 17 days' incubation
	0.1	Neutralization
	0.025	No neutralization
From three vaccinated monkeys which <i>succumbed</i> to nasal instillation of virus	0.8	Neutralization
	0.4	"
	0.1	Partial neutralization?—paralysis after 15 days' incubation
	0.025	No neutralization
From one similarly vaccinated monkey which <i>resisted</i> nasal infection on four attempts at monthly intervals but succumbed to an intracerebral injection of 0.5 cc. of 5% virus suspension	0.8	Neutralization
	0.1	"
	0.025	No neutralization
Normal monkey sera	0.8	" "
	0.8†	" "

* Total mixture made up to 1 cc. with saline, when necessary, and after incubation injected intracerebrally in a monkey.

† Only 0.1 cc. of a Berkefeld N filtrate of 5 per cent virus suspension was used in this mixture.

Correlation between Antibody and Susceptibility to Reinfection.—It is evident from the results shown in Table I that convalescent monkeys are resistant to reinfection at a time when their sera contain no demonstrable antibody. By the use of the same test, antiviral bodies were readily detected in the serum of vaccinated monkeys which proved to

sis and prior to reinoculation, in order to determine whether or not they possessed demonstrable antibodies at the time they were tested for resistance to reinfection (Table I).

The neutralization test was performed in the same manner as that previously used by Olitsky and Cox (2) for demonstrating antibody in the serum of vaccinated monkeys, whose resistance to infection is now being compared with that of the convalescents. In brief, 0.2 cc. of a Berkefeld N filtrate of a 5 per cent suspension of poliomyelitis cords in saline solution was mixed with 0.8 cc. of the serum, incubated 2 hours at 37°C., and overnight in the refrigerator, and the whole mixture injected intracerebrally in a monkey. The amount of virus in this mixture represented approximately twenty minimal infective doses.

It should be noted that none of the sera obtained from five monkeys had any demonstrable antibodies 4 to 5 weeks after the onset of paralysis; at 2 months the sera of only two of six monkeys tested failed to neutralize, while at 3 months these two also exhibited antiviral bodies. Many of the neutralization tests were repeated several times with the same results; hence it is clear that the development of antiviral bodies in convalescent monkeys is generally quite slow and at times may require as long as 3 months to become demonstrable. Reports of the presence of antibody as early as 36 hours after paralysis (8b) should, therefore, be regarded either as exceptional or as the result possibly of misinterpretation of a single test. Leake (14) reported the absence of neutralizing antibodies in a monkey 1 month after the onset of poliomyelitis, and Aycock and Kramer (15) found no antibody in two convalescent sera obtained 4 to 6 weeks after paralysis, although at 6 months after the disease the sera of these animals neutralized the virus.

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Correlation between Antibody and Susceptibility to Reinfection.—It is evident from the results shown in Table I that convalescent monkeys are resistant to reinfection at a time when their sera contain no demonstrable antibody. By the use of the same test, antiviral bodies were readily detected in the serum of vaccinated monkeys which proved to

be fully susceptible to the nasal instillation of poliomyelitis virus (2). It is clear, therefore, that the difference in resistance between convalescent and vaccinated monkeys is not directly related to the content of antiviral bodies. In order to study further the possible quantitative relationship between the humoral antibodies and resistance to nasal infection, the sera of three convalescent monkeys, bled after the development of demonstrable antibodies, were pooled and titrated simultaneously with the pooled sera of three vaccinated monkeys which failed to resist infection. The serum of another monkey (vaccinated at the same time and in the same manner as the other three) which resisted four different intranasal tests at monthly intervals but succumbed to an intracerebral inoculation of 0.5 cc. of a 5 per cent virus suspension was similarly titrated. Decreasing amounts of the various sera were added to a constant amount of virus, *i.e.*, 0.2 cc. of a Berkefeld N filtrate of a 5 per cent pooled cord suspension, the amount employed in all the other neutralization tests. The results, shown in Table II, indicate no appreciable quantitative difference in serum antibody in monkeys which resisted infection and in vaccinated monkeys which succumbed to the same intranasal test dose of poliomyelitis virus.

DISCUSSION

In view of the recently accumulated evidence which indicated that the majority of monkeys, treated with preparations of active virus, are not rendered resistant to nasal instillations of poliomyelitis virus in spite of the fact that they develop readily demonstrable serum antiviral bodies (1, 2, 3, 5), it was desirable to examine by similar methods the resistance and serum antibodies of monkeys recovering from a distinct paralytic attack of the experimental disease.

In recent years investigators who found vaccinated monkeys with serum antibodies and without resistance to intracerebral or intranasal infection with poliomyelitis virus postulated a certain "tissue immunity" as distinct from humoral immunity (1). It was not clear, however, to what extent variations in the quantitative level of antibodies in the serum could account for the difference in susceptibility or resistance to infection. Thus it may have been supposed that convalescent monkeys and those of the vaccinated ones which resisted infection might have had a larger amount of serum antibodies.

In the present investigation, nine convalescent monkeys were tested for susceptibility to infection with poliomyelitis virus by way of the nose. Three of these monkeys succumbed with unusual signs, but careful postmortem study eliminated a second attack of poliomyelitis as the cause of death; the remaining six successfully resisted repeated instillations of virus which in each case produced poliomyelitis in all the control monkeys. Of particular interest was the observation that convalescent monkeys were resistant to reinfection before antiviral bodies were demonstrable in their serum, and that the sera of all the monkeys tested several times 4 to 5 weeks after paralysis contained no demonstrable antibody; all monkeys, however, finally developed antibodies—some of them at 2 months and others not until 3 months after the onset of paralysis. It should be pointed out that by the use of the same test, vaccinated monkeys have been shown to contain readily demonstrable serum antibody at 5 to 6 weeks after the first inoculation without, however, exhibiting any resistance to the same amount of virus instilled intranasally (2). It was furthermore demonstrated that the serum of convalescent monkeys, when antibody finally appeared in it, was no more potent than that of the susceptible, vaccinated monkeys. It is interesting to compare these results with some of those recently reported by Jungeblut (9). His studies differed from these in that the virus was injected intracerebrally. He showed that of twenty-three convalescent monkeys studied at different times after the onset of paralysis, all resisted reinoculation with large doses of virus (no peculiar deaths of the type described here were reported) and this resistance was apparent long before the appearance of antibodies in the serum. It appears, therefore, that the resistance of convalescent monkeys to reinfection with the same strain of virus by either the intracerebral or intranasal routes cannot be correlated with the demonstrable presence of antiviral bodies in the blood. In order to avoid misleading generalizations from this observation, it should be recalled that different viruses may act differently in the same host, and that even the same virus may vary in this respect in two distinct hosts. Thus, the virus of equine encephalomyelitis readily and rapidly induces serum antibodies in *Macacus rhesus* monkeys, most of which do not become resistant to intracerebral inoculation of the virus (16), while in the guinea pig even completely inactivated, formolized vaccines give rise to an ex-

traordinary resistance to intracerebral inoculation (1000 M.I.D. or more) with very little or no antibody in the serum (17).

CONCLUSIONS

1. Monkeys convalescent from a paralytic attack of poliomyelitis develop humoral antibodies slowly; in the present series their first appearance in most was at 2 months and in some not until 3 months after the attack.

2. Convalescent monkeys display resistance to reinfection with the same strain by the nasal route long before antibodies become demonstrable in their serum, in this respect differing from many vaccinated monkeys whose serum neutralizes the virus, while they remain susceptible to nasal infection.

3. When antibodies appear in the serum of resistant convalescent monkeys, they are not quantitatively greater than in the serum of vaccinated monkeys which succumb to infection. As regards resistance to infection, humoral antibodies, therefore, do not have the same significance in vaccinated as in convalescent poliomyelitis monkeys.

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STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

VII. CULTIVATION OF THE COCCOBACILLIFORM BODIES IN FERTILE EGGS AND IN TISSUE CULTURES

By JOHN B. NELSON, Ph.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATE 41

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The regularity with which the minute Gram-negative bodies, tentatively designated coccobacilliform bodies, were found in films of exudate from birds experimentally infected with the coryza of slow onset was suggestive evidence that they were of etiological significance. This view was supported by the observed infectivity of nasal exudate which contained only the coccobacilliform bodies, no other agent being demonstrable microscopically, culturally, or by filtration (1). To establish a causal relation between these bodies and the coryza, however, it was essential that they be separated from exudate and obtained in a relatively pure state. Since growth had never been observed in nutrient media enriched with blood and other substances, it was evident that the usual cultural methods would be of no assistance. Centrifugation was attempted but proved to be impractical. Attention was finally directed to the procedures which have proved successful in the cultivation of the filterable viruses and the rickettsiae, namely: the egg membrane method of Woodruff and Goodpasture (2) and the tissue culture method of Li and Rivers (3). The present paper is concerned with the behavior of the coccobacilliform bodies in these media.

Cultivation of the Coccobacilliform Bodies in the Fetal Membrane of Fertile Eggs

Preliminary observations on the inoculation of fertile eggs in the 10 day stage, which are usually employed in cultivating the filterable

viruses, were disappointing. Most of the treated eggs continued to develop normally and when opened failed to show any trace of the bodies. It was subsequently found, however, that eggs in an earlier state of development, inoculated on about the 4th day of incubation, provided fairly satisfactory conditions for multiplication.

The initial inoculum used in all the following experiments was nasal exudate from infected birds of a group in which the coryza of slow onset had been maintained over a period of months by serial passage (4). While the exudate from the nares or the nasal passages of these birds almost invariably contains readily cultivable bacteria, that from the infraorbital sinuses not infrequently is sterile under ordinary cultural conditions. A presumptive bacteriological appraisal of exudate removed from the sinuses and suspended in saline was made by microscopic examination, the exudate being from birds which had shown a nasal discharge for only a few days. If Gram-stained films showed coccobacilliform bodies but no bacteria, approximately 0.1 cc. of the suspension was added to sterile defibrinated horse blood at the base of a nutrient agar slant. The culture was incubated at 37°C. for 24 hrs. If no bacteria were observed either microscopically or macroscopically, the suspension which had been kept meanwhile in the ice box was used for inoculation. Exudate which showed bacteria either in the preliminary films or in cultures was discarded.

The Inoculation of 10-11 Day Eggs

Fertile eggs which had been incubated for 10-11 days in a poultry incubator at 40°C. were first employed. A small window 8 x 10 mm. was cut through the outer shell using a diamond pencil and a pair of small, sharp pointed scissors. With a capillary pipette several drops of the exudate suspension were implanted on the fetal membrane and the opening sealed with vaseline and a sterile coverslip. The inoculated eggs were again incubated at 40°C. and examined daily to determine their condition. Activity of the living embryo was clearly visible through the coverslip. If there was no evidence of life the opening in the shell was enlarged sufficiently to remove the membrane about the site of inoculation. Films prepared by crushing small pieces of membrane in distilled water on a slide were Gram-stained, using carbolfuchsin diluted 1-4 with water as the counterstain.

Three of four 10 day eggs each contained a well developed living embryo when opened on the 5th day after inoculation. The fourth egg was opened on the 2nd day and showed a poorly developed inactive embryo. Coccobacilliform bodies were not found in films prepared from the membranes of the four inoculated eggs. Four 11 day eggs likewise failed to show the specific bodies in membrane

films. These eggs were all opened on the 8th day after inoculation. In three eggs the embryos, although large and well developed, were inactive at this time. They had, however, shown activity through the 7th day following inoculation. The embryo in the fourth egg was still alive when examined.

The Inoculation of 3-4 Day Eggs

In a preliminary experiment it was found that the inoculation of 3-4 day eggs was attended, in some cases, by the appearance of coccobacilliform bodies in the fetal membranes. A considerable number of inoculations were subsequently made in such eggs, generally 4 day eggs, to determine the distribution of the coccobacilliform bodies in the egg, their effect on the embryo, and whether successive egg to egg transfer could be made.

Three separate series of inoculations in 3-4 day eggs were conducted, in each case using exudate as the initial inoculum. Thereafter, transfers were made from egg to egg, inoculating with a suspension of finely divided membrane in 0.5 cc. of saline. Two to five eggs were inoculated with each passage. The membrane suspensions were tested for sterility by subculturing in horse blood agar. The inoculated eggs were incubated at 40°C. and examined daily. They were generally opened on the 3rd day when films were made from the membrane and in some cases from the amniotic fluid and the embryo. In particular instances histological sections were prepared from the membranes. The sections were cut to 5 μ and stained with phloxin-methylene blue.

In two series successful transfer of the coccobacilliform bodies was made through 6 consecutive passages and in the third series through 11 passages. Termination of the serial transfer was intentional in each case. Approximately 50 per cent of the inoculated eggs showed coccobacilliform bodies in the fetal membranes, the actual figures being 46 positive and 48 negative. A total of 94 eggs was employed in the three series and of these only six showed an active living embryo on the 3rd day after inoculation (usually the 7th day of life). The specific bodies were found in only one of the six eggs with live embryos.

In general the inoculated eggs fell into three groups. The first group comprised the eggs which showed when opened a well preserved and well developed embryo, with a transparent membrane and a clear or nearly clear amniotic fluid. In these eggs there was an indication

that the embryo had undergone some development following inoculation. 28 of the 46 positive eggs and 12 of the 48 negative eggs may be classed in this group. The second group comprised the eggs which gave indication of a prompt cessation of growth after inoculation. The embryo was small and less developed than those in the first group, the fetal membranes were opaque, and the amniotic fluid turbid. 18 of the positive and 21 of the negative eggs fell in this group. The third group comprised the eggs which showed a bacterial contamination coincident in most cases with extensive autolytic or digestive changes. This group included only a single positive egg but numbered 15 negative eggs.

Distribution of the Coccobacilliform Bodies in Inoculated Eggs

The coccobacilliform bodies in membrane films were identical morphologically with those in exudate but in general were considerably more numerous in the former case, only rarely being so sparse as to require a prolonged search. They were predominantly extracellular and commonly arranged in irregular or roughly spherical groupings which were frequently in close proximity to tissue cells. The bodies were also found as discrete or paired units. Occasionally they appeared to lie within tissue cells. In such cases, however, it would be difficult to prove that they were not on rather than in the cell.

The coccobacilliform bodies were never found in films made from the embryo even though they were numerous in the fetal membrane. Their certain detection in amniotic fluid was made difficult by the frequent presence of Gram granules of varying size, particularly in fluid from eggs which gave evidence of degenerative or autolytic changes. In most cases it seemed probable that the bodies were not present. Prolonged microscopic examination of films from horse blood agar slants liberally inoculated with membrane material regularly failed to reveal the specific bodies.

Pathological Changes in Inoculated Eggs

Macroscopically there were no obvious pathological changes which could be attributed to the coccobacilliform bodies. The fetal membranes of eggs which showed a prompt cessation of growth were

usually soft and cloudy but this change was no more pronounced in the presence of the bodies than in their absence.

The histopathology of membranes from inoculated eggs was not studied extensively. Sections from positive eggs with well developed embryos showed irregular ectodermal thickenings together with degenerative changes as well as necrosis of cells. Mononuclear cells were often encountered, particularly in the mesoderm. Occasionally there was an infiltration of cells resembling leucocytes. Similar though less extensive changes were also observed in the absence of the specific bodies. While these changes may be indicative of a reaction to traumatic injury, it seems probable that they are in part referable to the multiplying coccobacilliform bodies and that both factors contribute to the ultimate death of the embryo.

Extracellular and less commonly intracellular coccobacilliform bodies were generally found in sections of membranes which showed them in films. In some cases they were particularly conspicuous in the mesoderm where they evidently multiply in the intercellular spaces forming wreaths about the mesodermal cells. In sections stained with phloxin-methylene blue the specific bodies stain a deep blue.

Growth of the Coccobacilliform Bodies in 9 Day Eggs

A single additional experiment indicated that the coccobacilliform bodies were potentially capable of multiplying in older eggs if the conditions were favorable. Four 9 day eggs were inoculated with a saline suspension of membrane from 4th passage eggs in series 3. The eggs were opened on the 3rd day after inoculation at which time the embryo was inactive in each case. Two of the eggs showed numerous specific bodies in the fetal membranes. It seems probable that multiplication in these eggs is conditioned by an inactivation of the embryo brought about by other causes.

Cultivation of the Coccobacilliform Bodies in Tissue Culture

The preceding experiments clearly demonstrated that the coccobacilliform bodies of fowl coryza were cultivable in the fetal membranes of fertile eggs. Because of the uncertainty of obtaining a growth of the bodies upon inoculation, the method was not suitable

for routine cultivation. Accordingly, attention was directed towards growing the bodies in tissue culture.

The method employed was essentially that of Li and Rivers; using, however, test tubes instead of flasks. Traub (5) had successfully employed tubes in the cultivation of pseudorabies virus as had also Dochez, Mills, and Kneeland (6) in the case of the common cold virus of man. The technic which was finally adopted entailed the suspension of approximately 75 mg. of finely minced 10 day chick embryo tissue (the eyes were removed from the embryos before mincing) in 5.0 cc. of Seitz-filtered Tyrode's solution in test tubes with an inside diameter of approximately 15 mm. Since these tubes were not selected for size, the height of the column of fluid varied somewhat but was in the vicinity of 3.5 cm.

Freshly prepared tissue culture medium was usually incubated for 24 hours at 37°C. prior to use. Bacteria grow vigorously in the medium and if contaminants are present a clouding of the supernatant is generally apparent after a day's incubation. Observing ordinary aseptic precautions, little difficulty was experienced with contaminated tubes. The medium was commonly prepared twice a week; one embryo providing sufficient material for 15-18 tubes.

In carrying stock cultures of the specific bodies a liberal inoculum was used, several drops of fluid containing tissue fragments being added. The inoculated tubes were examined after 24 and 48 hours at 37°C., films being made from the supernatant as well as the sedimented tissue and Gram-stained.

Two isolations of the coccobacilliform bodies were made from exudate and the cultures maintained over a period of weeks by successive subinoculations at intervals of 24 to 72 hours. The first strain was isolated in November, 1935, and has been carried through 100 generations. The second strain originally obtained in March, 1936, is now in its 37th subculture.

Distribution of the Coccobacilliform Bodies in Tissue Cultures

The specific bodies are readily found in the sedimented tissue of an inoculated culture tube and also in the supernatant, regardless of whether or not the tube has been disturbed since inoculation. Growth is generally apparent after 24 hours' incubation but occasionally is delayed until the 2nd day. In the fluid portion of the medium the specific bodies are discrete or in small groupings. In a 24 hour culture they are well stained and sharply outlined, being readily differentiated from extraneous granular material. The bodies are rather sparsely distributed in the supernatant but appear nevertheless to impart a very faint turbidity to it. Whether this turbidity is due solely to the

suspended bodies or in part to material derived from the tissue fragments as the result of autolytic changes is uncertain. A difference between the supernatant turbidity of inoculated and uninoculated tubes is detectable only in freshly prepared media. In media which have been stored for several days and then incubated, the supernatant acquires a distinct cloudiness as the result of tissue disintegration.

While the coccobacilliform bodies unquestionably multiply in the nearly cell-free supernatant of inoculated tissue cultures, the chief site of multiplication is in the sedimented tissue fragments. Tissue films from a 24 hour culture generally show numerous bodies as discrete units or in groups of varying size. They are frequently grouped around tissue cells or embedded in what appears to be disintegrated tissue. They may also appear to lie within intact tissue cells, large groupings of which are often present. In this case they are to all appearances intracellular. It seems unlikely that they have simply been caught by the cells in preparing the film. If the medium is inoculated by adding a drop of culture to the supernatant without shaking, multiplication of the bodies in the sedimented tissue may be delayed.

Fragility of the Coccobacilliform Bodies in Tissue Culture

In tissue cultures the coccobacilliform bodies quickly lose their staining affinity and sharpness of definition and become faintly stained particles with a hazy outline which are hard to differentiate from extraneous granules. This change may occur as early as the 3rd day in cultures which have shown numerous well defined bodies after 24 hours of incubation. Such cultures may, however, retain their viability for a week or longer if kept at ice box temperature and give rise to a new generation of deeply stained and sharply outlined bodies upon transfer to fresh tissue culture medium.

Identification of the Coccobacilliform Bodies in Fetal Membranes and in Tissue Culture

Comparatively little difficulty has been experienced in the microscopic identification of the coccobacilliform bodies either in the fetal membranes of fertile eggs or in tissue cultures. As previously noted, the presence of Gram granules of varying size in eggs which show

autolytic or disintegrative changes may constitute a source of confusion. In well preserved membranes and in freshly prepared tissue cultures there are surprisingly few particles which might be mistaken for the specific bodies. There are encountered, from time to time, in freshly prepared tissue culture medium spherical cells of small size but averaging somewhat larger than the coccobacilliform bodies. These granules are weakly Gram-positive but some of them are generally decolorized. They may be discrete or in small groups, are generally extracellular but may also be intracellular, and vary in numbers from few to many. Usually they are not present in all the tubes of a given lot of medium and often are not encountered at all. It seems probable that they are cell granules of some kind as there is no indication that they are transferable in series or cultivable in other media. In spite of their staining reaction they may constitute a source of error for an inexperienced observer.

DISCUSSION

Multiplication of the coccobacilliform bodies of fowl coryza outside the host is clearly established by the experiments here reported. Suitable environmental conditions for growth are supplied by the fetal membranes of fertile eggs in about the 4th day stage and by tissue cultures.

Unlike the rickettsiae and the filterable viruses the fowl coryza bodies are not readily cultivable in the membranes of 10 day eggs. The membrane relations of eggs in these respective stages of development are quite different. In the 10 day egg the fetal membrane comprises an outer chorionic and an inner allantoic portion. In the 4 day egg the allantoic membrane has just begun to develop and essentially only a chorion is present. Woodruff and Goodpasture (2) noted that membrane inoculation with fowl pox virus in embryos younger than the 6 day stage was usually unsuccessful. The injuries attendant on inoculation generally resulted in an early death of the embryo. This effect was well brought out in the present series in which only five of 94 inoculated eggs, in the 3-4 day stage, showed living embryos on the 3rd day of incubation in spite of the fact that the specific bodies failed to develop in 48 per cent of them. It seems probable that sufficient opposition is encountered in the vigorously

growing 10 day egg to prevent multiplication of the introduced bodies and that growth does occur only when the embryo is accidentally inactivated. The greater frequency of multiplication in 4 day eggs may well be correlated with the greater susceptibility of such eggs to traumatic injury with subsequent inactivation of the embryo.

Death of the embryo does not necessarily imply the immediate death of its component cells or those of the supporting fetal membranes. There was a suspicion, however, from the behavior of the coccobacilliform bodies in inoculated eggs that living cells were not essential for their growth as in the case of the rickettsiae and the filterable viruses. There was also a suggestion from the manner in which the specific bodies multiplied in tissue cultures that they were capable of independent growth in a suitable fluid medium in the absence of living cells. These observations which have been confirmed and extended by other experiments, to be reported later, appear to preclude the classification of the coccobacilliform bodies with either the rickettsiae or the filterable viruses.

SUMMARY

The coccobacilliform bodies of fowl coryza were successfully cultivated in the fetal membranes of fertile eggs. Microscopic examination indicated growth in approximately 50 per cent of 94 eggs inoculated on the 3rd to 4th day of incubation. Growth was generally inhibited, however, in eggs inoculated on the 10th day. One strain of the specific bodies was maintained through 11 successive passages in 4 day eggs.

A more consistent growth of the coccobacilliform bodies was obtained in tissue cultures. One strain, originally isolated in November, 1935, has been carried through 100 successive subcultures at intervals of 1-3 days. The specific bodies fail to maintain their morphological identity for any length of time in this medium.

It is noted that growth of the coccobacilliform bodies in fertile eggs and in tissue cultures is not dependent on the presence of living cells.

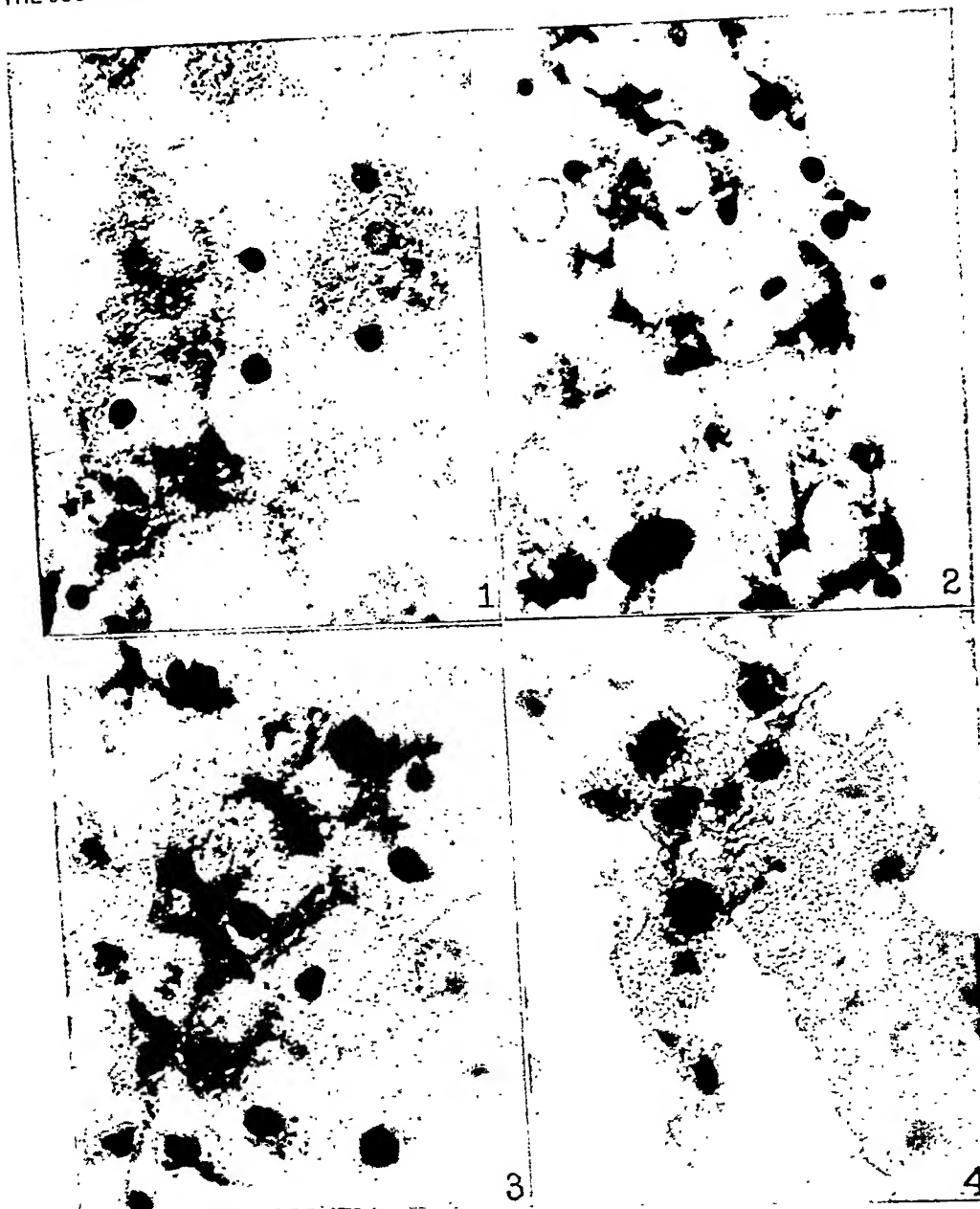
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EXPLANATION OF PLATE 41

- FIG. 1. Extracellular coccobacilliform bodies in the fetal membrane of an inoculated 4 day egg. Gram-stained film. $\times 920$.
- FIG. 2. Coccobacilliform bodies outlining mesodermal cells in fetal membrane of an inoculated 4 day egg. Section (5μ) stained with phloxin-methylene blue. $\times 920$.
- FIG. 3. Intracellular coccobacilliform bodies in tissue fragment from a 24 hour tissue culture. Gram-stained film. $\times 920$.
- FIG. 4. Coccobacilliform bodies in disintegrating tissue from sediment of a 24 hour tissue culture. Gram-stained film. $\times 920$.



Photographed by J. A. Carlile

(Nelson: Uncomplicated coryza of domestic fowl. VI)

STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

VIII. THE INFECTIVITY OF FETAL MEMBRANE AND TISSUE CULTURE SUSPENSIONS OF THE COCCOBACILLIFORM BODIES

By JOHN B. NELSON, Ph.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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The successful cultivation of the coccobacilliform bodies of fowl coryza in the fetal membrane of fertile eggs and in tissue cultures provided an opportunity to determine their effect on the mucosa of the upper air passages when introduced in a medium which did not contain exudate.¹ Accordingly, infectivity tests were conducted in normal fowl with suspensions prepared from such cultures. The results of these tests, together with observations on the transmissibility by injection and the communicability by direct contact of the resulting coryza, are herewith reported.

The Infectivity of Fetal Membrane Suspensions

The fetal membranes employed in this experiment were from fertile eggs included in the three inoculation series referred to in the preceding paper.¹ Normal birds were injected intranasally with suspensions of these membranes.

Suspensions were prepared only from membranes which showed the specific bodies in the absence of bacteria. Small pieces of the chorion from inoculated eggs were crushed in a glass tissue grinder and the finely divided tissue suspended in 2-3 cc. of saline. Normal birds, 2-3 months old, were injected intranasally by way of the palatine cleft with approximately 0.5 cc. of these suspensions. Four birds were usually employed at a time. They were maintained in single cages in a special unit, under strict quarantine, and were examined daily. Birds which showed a nasal discharge were generally autopsied within a week of its

¹ Nelson, J. B., *J. Exp. Med.*, 1936, 64, 749.

appearance. In the absence of symptoms the birds were killed after an observation period of 3-6 weeks. At autopsy, in both cases, the upper air passages and trachea were exposed and examined. Films, which were Gram-stained, were made from the nasal chambers and, if infected, from the infraorbital sinuses as well. In particular cases, tissue from the nasal mucosa was sectioned and blood agar cultures were made from the exudate.

A summary of the data pertaining to the birds injected with egg membrane suspensions is presented in Table I. It may be noted that 4 of the birds in series 3 received suspensions from 11th passage eggs.

Fourteen of the 24 birds in this experiment showed a nasal discharge following injection. One bird developed a conjunctivitis with a foamy lacrimation but had no discharge from the nares. In Table I, as also in the subsequent tables, this form of coryza, which is mani-

TABLE I
The Intranasal Injection of Coccobacilliform Bodies in Fetal Membrane Suspension

Egg series	Birds injected	Cases of apparent coryza	Cases of in-apparent coryza	Normal birds	Specific bodies in films
1	8	2	5	1	7
2	8	5	2	1	6
3	8	8	—	—	8
Total.....	24	15	7	2	21

fest by nasal or orbital symptoms during the life of the bird, is termed apparent coryza. 7 of the injected birds showed no coryzal manifestations during life but at autopsy there was a well marked mucopurulent or catarrhal inflammation of the nasal chambers and usually of the infraorbital sinuses, in addition. This form of coryza, which is characterized by inflammatory changes detectable only at autopsy, is termed inapparent coryza. Two of the injected birds showed no indications of coryza either during life or at autopsy.

Six of the 14 birds which showed nasal symptoms had a bilateral discharge, 8 a unilateral. The interval between the injection of exudate and the appearance of a discharge varied from 13-27 days. In only 4 cases, however, was the incubation period longer than 17 days. 13 of these birds had both a rhinitis and a sinusitis, one a rhinitis only. All of them showed coccobacilliform bodies in

films from either the nasal passages or the sinuses and 3 showed miscellaneous bacteria in addition.

Each of the 7 inapparent cases of coryza showed a rhinitis at autopsy; 5 of them also had a sinusitis. In 6 cases coccobacilliform bodies were present in films from either the nasal passages or the sinuses. In only one instance were miscellaneous bacteria also observed.

TABLE II

The Intranasal Injection of Coccobacilliform Bodies in Tissue Culture Suspensions

No. of culture generation	Bird No.	Cases of apparent coryza	Cases of inapparent coryza
18	1	+21*	
	2	—	+
	3	—	+
	4	+14	
36	5	+17	
	6	—	+
	7	—	+
	8	—	—
55	9	+12	
	10	+10	
	11	+9	
	12	—	+
72	13	+15	
	14	—	+
	15	—	+
	16	—	+
87	17	+23	
	18	—	+
	19	—	+
	20	+13	
Total.....	20	9	11

* The number in this column and in the tables which follow indicates the days between injection and the appearance of a nasal discharge.

The Infectivity of Tissue Culture Suspensions

Five generations—the 18th, 36th, 55th, 72nd, and 87th—of a strain of the coccobacilliform bodies originally isolated in November,

1935, and maintained thereafter in tissue cultures were tested for infectivity by intranasal injection in normal fowl.

The supernatant was removed on the 1st or 2nd day of incubation from tissue cultures which showed numerous coccobacilliform bodies in films. The sedimented tissue was transferred to a glass tissue grinder, finely triturated, and suspended in 2 to 3 cc. of the same supernatant. 4 normal birds were injected by way of the palatine cleft with 0.5 cc. amounts of such suspensions, from each of the 5 generations. The procedure followed in the maintenance and examination of these birds was identical with that employed in the preceding experiment.

As indicated in Table II a coryza was produced in all the birds which received a nasal instillation of the tissue culture suspensions. In only 9 cases, however, was the coryza of the apparent type, manifested by a nasal discharge. 11 of the birds showed an inapparent coryza with a nasal inflammation detectable only at autopsy.

Seven of the 9 cases of apparent coryza showed a unilateral nasal discharge and 2 a bilateral. The incubation period varied from 9 to 23 days. At autopsy there was a definite rhinitis as well as a sinusitis in all of these birds. In each case the specific bodies were present in nasal films. 4 of the birds also showed miscellaneous bacteria.

Three of the birds with the inapparent form of coryza were autopsied during the 3rd week after injection, the others after the 4th week. Inspection of the nasal chambers showed a well marked inflammation with considerable free exudate in every case. 10 of the birds also had a bilateral or unilateral sinusitis. All of the birds showed coccobacilliform bodies in nasal films but in only 3 were miscellaneous bacteria also present.

Serial Transmission by Injection of the Coryza Produced by Fetal Membrane and Tissue Culture Suspensions

Two experiments were carried out to determine whether the coryza produced by fetal membrane and tissue culture suspensions, respectively, could be transmitted serially by the intranasal injection of exudate.

A summary of these experiments is given in Tables III and IV. A coryza originally produced in a susceptible bird by the introduction of a fetal membrane suspension was maintained for 7 successive passages by the transfer of exudate from infected to normal fowl, one bird being employed for each passage. The bird injected with the initial membrane suspension and 3 of the birds which received exudate

showed a coryza of the apparent type with a delayed nasal discharge. In 4 of the birds the coryza was of the inapparent type characterized

TABLE III

The Serial Transfer of Exudate Originally Produced by Fetal Membrane Injection

Material injected	Bird No.	Cases of apparent coryza	Cases of inapparent coryza
Fetal membrane suspension.....	1	+13	
Exudate from 1.....	2	+15	
" " 2.....	3	+19	
" " 3.....	4		+
" " 4.....	5		+
" " 5.....	6		+
" " 6.....	7	+11	
" " 7.....	8		+
Totals.....	8	4	4

TABLE IV

The Serial Transfer of Exudate Originally Produced by Tissue Culture Injection

Material injected	Bird No.	Cases of apparent coryza	Cases of inapparent coryza
Tissue culture suspension	1		+
	2		+
Exudate from 1 and 2	3		+
	4		+
Exudate from 3 and 4	5		+
	6	+23	
Exudate from 5 and 6	7		+
	8	+21	
Exudate from 7 and 8	9		+
	10	+20	
Totals.....	10	3	7

by a rhinitis and in 3 cases by a sinusitis. Coccobacilliform bodies were regularly present in nasal films. This series was discontinued after the 7th passage.

A coryza originally produced by the 36th generation of the specific bodies in tissue culture was similarly maintained for 4 passages, using, however, 2 birds with each transfer. The 2 birds infected by the original tissue culture suspension developed a coryza of the inapparent form, both of them showing a rhinitis and sinusitis at autopsy. In 3 of the birds injected with exudate the coryza was of the apparent form with a delayed nasal discharge. 4 of the birds showed manifestations of coryza only at autopsy, there being a rhinitis in each case and a sinusitis in 3 cases. Coccobacilliform bodies were present in nasal films from all of these birds. This series of passages is being continued.

Communicability of the Coryza Produced by Fetal Membrane Suspensions

Two contact experiments were carried out to establish the communicability of the coryza produced by the nasal injection of fetal membrane suspensions.

In conducting these experiments it was necessary to employ birds injected with exudate from cases of coryza originally established by fetal membrane injection. Only apparent cases of coryza with a well marked nasal discharge of several days duration were used. In the first experiment 4 infected birds and in the second experiment 6 birds were placed in the same pen (approximately 7 feet long and 5 feet wide) with equal numbers of normal fowl of the same age. Unless the susceptible birds showed a nasal discharge at any earlier time, the two groups were kept together until the experiment was ended. The birds which did show a nasal discharge were autopsied within several days of its appearance. At the end of the observation period, the remaining birds were killed and examined. The first contact experiment was begun the latter part of November, 1935, and the second early in January, 1936.

The results of the two contact experiments are summarized in Table V. One of the 4 susceptible birds in the first series developed a coryza of the apparent form, with a nasal discharge which was first observed on the 32nd day of confinement. 3 of the birds, killed on the 36th day of confinement, showed a coryza of the inapparent form with an inflammation only of the nasal chambers. Coccobacilliform bodies were present in nasal films in each case. All of the susceptible birds in the second series developed a coryza of the apparent type, the incubation period varying from 18-24 days.

Examination of the upper air passages, at autopsy, revealed an inflammation of the nasal chambers in each case. 5 of the birds also had a sinusitis. Each of the 6 birds showed coccobacilliform bodies in nasal films and 5 of them numerous miscellaneous bacteria in addition.

Three of the originally infected birds in the first series and 2 in the second ran an intermittent nasal discharge during the entire period of confinement. In the remaining birds the discharge was continuous but tended to fluctuate from a unilateral to a bilateral form. One of the contact birds in series 2 developed a unilateral conjunctivitis in addition to a nasal discharge.

TABLE V

Communicability of the Coryza Produced by Fetal Membrane Suspensions

No. of series	No. of birds in contact	Cases of apparent coryza	Cases of inapparent coryza
1	1	+32	+
	2		
	3		+
	4		+
2	5	+20	
	6	+20	
	7	+24	
	8	+21	
	9	+20	
	10	+18	
Totals.....	10	7	3

For purposes of comparison the results of an additional contact experiment may be presented. This experiment was carried out with birds injected with exudate from fowl in which a strain of the coryza of slow onset has been maintained for 2 years by serial passage.² Contact between the infected and the normal birds, which numbered 5 each, was established early in October, 1935.

All of the susceptible birds showed an apparent coryza with a frank nasal discharge, the respective incubation periods being 28, 26, 16, 22,

² Nelson, J. B., *J. Exp. Med.*, 1936, 63, 509.

and 26 days. Postmortem examination made within several days of the appearance of a discharge revealed a rhinitis in each case and a sinusitis in three cases. All of the birds showed coccobacilliform bodies as well as miscellaneous bacteria in nasal films.

Each of the originally infected birds maintained a continuous bilateral discharge during the period of contact. One bird also developed a bilateral conjunctivitis.

TABLE VI

Nature of the Coryza Produced by Bacteria-Free Suspensions of the Specific Bodies

Bird No.	Material injected	Incubation period	Duration of coryza	Nature of discharge
		<i>days</i>	<i>days</i>	
1	Exudate suspension	15	>62	Continuous
2	" "	13	>38	"
3	" "	20	>79	"
4	" "	15	>72	"
5	" "	10	>43	"
6	" "	22	>60	"
7	" "	14	50	Intermittent
8	" "	17	47	"
9	" "	25	20	Continuous
10	" "	22	>60	Intermittent
11	" "	13	>37	Continuous
12	Fetal membrane suspension	17	42	"
13	" " "	12	>66	Intermittent

The Incubation Period and Duration of the Coryza Produced by Bacteria-Free Suspensions of the Coccobacilliform Bodies

Comparatively little information is available concerning the incubation period and the duration of the coryza incited by the intranasal injection of fetal membrane and tissue culture suspensions. Frequently the coryza was of the inapparent type with inflammatory manifestations only at autopsy. Most of the birds which did show a nasal discharge were brought to autopsy within a week of its appearance.

A summary of the information at hand, derived from observations on 13 birds which were used in contact experiments, is given in Table VI. 2 of these birds were injected with fetal membrane suspensions

and 11 with nasal exudate from similarly infected fowl. The interval between the injection of the infective material and the appearance of a discharge varied from 10-25 days. Shortly after the onset of a discharge these birds were placed in contact with normal fowl and held under observation for a period of weeks. 4 of the birds recovered after a discharge which lasted 50, 47, 42, and 20 days, respectively. The other birds, which were autopsied prior to recovery, all showed a persistent discharge, in 6 cases being apparent for a period of at least 2 months. In 4 birds of this series the nasal symptoms were intermittent, in 9 continuous during the period of observation.

DISCUSSION

Fetal membrane and tissue culture suspensions of the coccobacilli-form bodies are manifestly infective for normal fowl. Only 2 of 44 birds which were injected intranasally with these suspensions failed to show a nasal inflammation at autopsy. The coryza produced in this way is serially transmissible by the nasal instillation of exudate and is fully as communicable by direct contact as the original strain of coryza which has been maintained by exudate passage. The information at hand concerning its duration is somewhat meager but indicates a similar tendency towards chronicity.

The reported observations do, however, indicate one characteristic which distinguishes it from the original strain of coryza. Infected birds frequently fail to develop a nasal discharge regardless of the period of confinement. This inapparent form of coryza is observed not only in birds injected with the fetal membrane and tissue culture suspensions but also in birds which have received exudate from fowl originally infected with these suspensions. The incidence of inapparent coryza in a group of 59 birds was 47 per cent. The inapparent form of coryza is characterized by a well marked rhinitis and generally a sinusitis which are demonstrable only at autopsy. It should be emphasized that similar inflammatory manifestations have never been observed in birds injected intranasally with sterile solutions as saline, nutrient bouillon, and blood bouillon or with suspensions made from uninoculated fetal membranes and tissue cultures.

A nasal discharge has appeared so regularly following the injection of exudate from birds infected with the original strain of coryza that

it has come to be regarded as a regular feature of the infection. During the 2 years that this strain has been maintained by serial passage, only 2 of 93 infected birds have failed to show a nasal discharge. Birds affected with the apparent form of coryza generally show a more copious exudate in the nasal passages, at autopsy, than do the birds with inapparent coryza.

At present this discrepancy cannot be definitely accounted for. There is, however, one outstanding difference between the respective suspensions used for injection. Tissue cultures and fetal membranes contain only the coccobacilliform bodies, whereas exudate from the birds infected with the original strain of coryza invariably contains numerous secondary bacteria in addition. It is often noted, too, that nasal exudate from fowl infected with fetal membrane and tissue culture suspensions harbors few cultivable bacteria or may even be bacteria-free.

These observations which indicate that the coccobacilliform bodies are the specific incitants of the nasal inflammation also offer a suggestion that this reaction may be modified nonspecifically by the presence of numerous miscellaneous bacteria. The bacterial flora of the normal nasal mucosa is quantitatively meager. Moreover, the few bacteria which are initially present generally do not multiply to any great extent in the advent of an inflammation and the secretion of exudate. With the injection of exudate derived from the original strain of coryza, however, the bacterial flora of the nasal tract is greatly augmented. Many of the introduced bacteria apparently survive and later multiply actively in the exudate which forms. The additive irritation exerted by these secondary bacteria might well account for the more copious secretion of exudate which is characteristic of the original strain of coryza. There is no direct proof, however, that the secondary bacteria do act as irritants and a final appraisal of the situation must await the outcome of controlled experiments on the action of the coccobacilliform bodies in the presence of these bacteria.

SUMMARY

Fetal membrane and tissue culture suspensions of the coccobacilliform bodies are infective for normal fowl. Intranasal injection is

commonly followed by a coryza which is serially transmissible and communicable by direct contact. The specific bodies are generally demonstrable in the nasal exudate of birds infected either by injection or contact.

Compared with the original strain of the coryza of slow onset the reaction produced by these suspensions is often less vigorous; the incidence of apparent cases, characterized by a nasal discharge, being 97 per cent and 53 per cent, respectively. The apparent cases are similarly characterized by a long incubation period and a tendency towards chronicity.

THE STANDARDIZATION OF LONGEVITY AGAINST DOSE IN EXPERIMENTAL TUBERCULOSIS BY INTRACEREBRAL INOCULATION

By KENNETH C. SMITHBURN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 42 TO 44

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Progress in experimental investigation of tuberculosis has been retarded by the chronicity of the disease and by the lack of regularity in the course pursued in experimental animals. Whereas in some other infectious diseases it has often been possible to control factors of dosage of culture and host resistance so that a standardized result was obtainable, such has not been the case in tuberculosis. Final results in animal experiments on tuberculosis have not been obtainable in less than a few months, and not infrequently a single experiment has covered a year or more. Thus, in our experience, in ten experiments including 120 rabbits, each inoculated intravenously with a standard dose of 0.1 mg. of B-1 bovine strain tubercle bacilli (then only moderately virulent) and subjected to no therapeutic procedure, the first animals succumbed in the 3rd week after inoculation (15 to 20 days) and several survived more than a year, while one animal lived 738 days. In nine of the ten experiments, maximum survival ranged from 290 per cent of minimum survival up to 4500 per cent. Our results with other, more virulent bovine strains and with the H-37 human strain have been similarly variable, although somewhat less so. Obviously such variations have made interpretation of results difficult and permitted unavoidable intercurrent factors to play a part in the end-result.

In order to overcome these difficulties, Bogen (1) has adopted the procedure of killing all inoculated animals after about 90 days and making quantitative estimates of the amount of existing disease. Animals dying before this time were excluded as having died from or

with extraneous causes. He has shown that his method is adaptable to statistical analysis, especially when very large numbers of animals are used. Even with such procedure, however, the time necessary for a single experiment is great, and the cost of animal maintenance also large.

It is readily apparent from the facts just mentioned that a standardizable infection would be a valuable new tool in tuberculosis research. It was toward this end that the experiments to be reported were directed. It was considered that the optimum result in untreated cases would be invariably death, preferably in a short time after inoculation, that a significant result should be obtainable with minimal doses of bacteria, and that all individuals of comparable stock similarly treated should succumb at approximately the same time. Having adopted these standards, three avenues of procedure were apparent: (a) search for a highly susceptible animal host; (b) use of an adjuvant (mucin) to enhance the virulence of tubercle bacilli; and (c) study of various routes of inoculation to determine which gave the most uniform result. Suffice it to say that all our experiments in the first two categories were unsatisfactory; but it was found that the intracerebral route of inoculation afforded a method for obtaining a standardizable result in guinea pigs.

During recent years several investigators have employed the intracerebral route of inoculation in their studies on tuberculosis. Krause (2) made intrathecal injections by the postorbital route in his studies on hypersensitiveness. His method was used by others in subsequent experiments (12).

Manwaring (3) trephined the skulls of dogs, inserted a paraffin plug, allowed the wound to heal, and made inoculations through the paraffin plug. He did no titration experiments but found that injections of leucocytes prolonged both the incubation period and survival time. Austrian (4) inoculated rabbits by a method similar to that of Manwaring (3) and also by the lumbar intrathecal route. Austrian's description of the clinical course and macroscopic necropsy findings is among the best on record. He suggested use of the method for diagnostic purposes. A few years later, Foot (5) introduced tubercle bacilli intracerebrally in rabbits to study the formation of lesions in the meninges. Kasahara (6) introduced tubercle bacilli into the subarachnoid space through the atlanto-occipital membrane and studied the chemistry and cytology of the cerebrospinal fluid. He called attention to the similarity of the disease so produced to the clinical infection in man. Soper and Dworski (7, 8) inoculated normal and previously vaccinated rabbits with varying doses of viable tubercle bacilli. They inoculated also

through the atlanto-occipital membrane. They found that superinfection of vaccinated animals with large doses resulted in early death, whereas when the superinfecting dose was small (8), the animals lived longer, demonstrating a protective effect of the primary inoculation. Bickford (9) also used the cisternal route of inoculation, making the observation that with small doses of organisms (800) the incubation period was longer than after a larger dose (500,000). A very interesting observation was made in 1929 by Shope and Lewis (10) and Lewis and Shope (11). They found a high incidence of paralysis in a group of guinea pigs inoculated subcutaneously with a strain of human tubercle bacilli isolated from sputum. They transmitted the disease in guinea pigs by serial intracerebral inoculations of brain suspensions, showed that the paralysis was caused by the tubercle bacilli, gave a good account of the clinical and pathological course of the disease, and described certain changes in properties of the organisms incident upon their residence in the central nervous system. The apparent special affinity of their strain of organisms for nervous tissue was unexplained.

An outstanding fact regarding the reports summarized above is that none of the investigators mentioned has done titration experiments and that for the most part very large doses of organisms were used. Several workers made note of the fact that animals of a group receiving the same dose died at approximately the same interval after inoculation (4, 5, 7). Moreover, all these workers indicated, as does Calmette (12) that the infection induced by intracerebral inoculation is acute and uniformly fatal. The experiments reported in the following paragraphs indicate also that it is a readily standardizable infection by means of which greater precision may be introduced into tuberculosis research.

Materials and Methods

Bacteria.—Six strains of tubercle bacilli were used in the experiments. They were: avian TS strain, isolated in 1933 by the late Dr. Theobald Smith; bovine strain 36, isolated from a cow in April, 1929, also by Theobald Smith; bovine strain B-1, isolated by Dr. E. R. Baldwin at Saranac in 1904; human strain H-37, also isolated by Dr. E. R. Baldwin in 1905; and human strains O'Donnell and Fox, isolated from human patients in 1935 by the author. Each of these strains, except the bovine B-1, was known to be pathogenic for susceptible animals. The B-1 strain, formerly virulent, is now almost wholly avirulent. Relatively young stock cultures of each strain grown on Corper's medium (13), adjusted to pH 6.8, were employed. Suspensions were prepared by first weighing the organisms immediately after removal from the tube, then grinding in a mortar with sterile physiological saline sufficient to make 1 mg. (moist weight of bacteria) per cc.

Dilutions were then prepared so that 0.1 cc. contained the desired number of organisms. When mucin was employed, only the final decimal dilution was made with mucin; the suspension therefore contained 90 per cent of the mucin preparation.

The heat-killed suspension of H-37 was prepared in the usual manner, placed in a tube of 50 cc. capacity, the top of which was well heated in a Bunsen flame, the tube sealed with a rubber cap, immersed in a water bath well beyond the level of the fluid suspension, and heated for 30 minutes while the bath was vigorously boiling.

Animals.—Guinea pigs purchased in the open market were used throughout. They were closely inspected and only vigorous, normal animals were selected. In most instances they were isolated for a few days prior to the experiment and observed for evidence of any disease. Male animals have been used for the most part, although females were also used in one experiment for comparison. Weights of the animals varied from 350 to 740 gm., the average being 479 gm. in the two larger experiments. In the quantitative experiments, groups of animals with closely matched individual and total weights were used, the average in one experiment being 420 gm. and in another experiment, 505 gm. per animal. Young animals weighing about 400 gm. were found to be most suitable for the trephining operation as the skull bones were soft enough to permit this operation to be done very easily.

Mucin.—In our first experiment with intracerebral inoculation of guinea pigs, half the animals were inoculated with organisms suspended in saline, the remainder with organisms suspended in mucin. The latter was prepared by Dr. G. Rake of The Rockefeller Institute, according to the method described by him (14). The bacteria were suspended in mucin by placing one volume of bacterial suspension and nine volumes of mucin in a Petri dish and stirring with a sterile rod or pipette.

Technique of Inoculation.—The hair is clipped from the scalp of the animal. Ether is administered to the point of full surgical anesthesia. The scalp is then painted with full strength tincture of iodine. Using aseptic procedure, a longitudinal incision, about 8 to 10 mm. long, is made in the skin about 3 mm. to the left of the midline. At a point in this plane not exceeding 4 mm. posterior to a line connecting the posterior commissures of the eyes, the skull is trephined. The instrument we have used is a No. 60 carpenter's drill fixed in a carpenter's hollow-handled pin vise,¹ the drill being so fixed in the chuck of the vise that the latter prevents penetration beyond the desired point. The injection is made from a tuberculin syringe fitted with a $\frac{3}{8}$ inch, 27 gauge needle, the amount injected being 0.1 cc. Closure of the skin incision is made with a single metal clip. The animals regain consciousness within a few minutes and, beyond being a little listless, show no untoward effects. With adequate assistance and a little experience, the entire operation requires less than a minute after the animal is anesthetized. The skin

¹ L. S. Starrett and Co., Athol, Mass., No. 162 B. The No. 60 drill has a diameter of 0.04 inch or approximately 1 mm.

clip is shaken out by the animal when the wound is healed. Infection of the wound is not encountered if the closure is properly done. An occasional animal shows torticollis and circular movements (two in 226 instances to date) but recovery is prompt and complete. We have had two deaths from operative trauma among 226 animals, so that the hazard of the method itself is small. Death from intercurrent disease or extraneous causes has occurred seven times in the same groups of animals: twice from peritonitis due to perforation of the gut while taking the temperature, twice from purulent meningitis (both in cases in which the skin wound was improperly closed), and three times from streptococcal pneumonia.

Pathological Examinations.—All animals were either allowed to die or were killed with chloroform when it was evident that exitus was but a matter of a few hours. Surveys of the pathology in the visceral organs and brains were made in the gross, the latter then being fixed in 10 per cent formalin and the other tissues in Helly's fluid. Paraffin sections were then prepared in the usual manner and stained with hematoxylin and eosin. Sections were stained for bacteria with hematoxylin and anilin-fuchsin and counterstained with light green. Sections of each hemisphere of the brain were cut in the sagittal plane. Estimates on the basis of 0 to ++++ were made of the extent of macroscopic and microscopic pathology and of the number of bacteria in the tissues. Sections of brain and spleen were examined as routine for the presence of bacteria, the other tissues only when it was especially desirable to do so.

EXPERIMENTAL

First Experiment.—Purpose: (a) to determine the acuity of infection induced by intracerebral inoculation of a standard dose of virulent avian, bovine, or human tubercle bacilli; (b) to ascertain whether mucin enhances the pathogenic properties of either of these organisms when introduced into the brain of guinea pigs.

Twelve guinea pigs were used in the experiment. Four individuals received avian, four bovine, and four human type organisms. Of the four inoculated with each strain, two received organisms suspended in saline, and two received the organisms suspended in mucin. In this experiment the dose of each organism was 0.15 mg. in 0.15 cc. of fluid introduced intracerebrally as described above.

Results.—Each of the twelve animals succumbed to the disease, as shown in Table I. The time of death was very irregular in the case of the four animals inoculated with avian organisms, namely, from 11 to 49 days. This was not surprising, however, as the susceptibility of guinea pigs to infection with the avian type bacilli is very low. The animals which received mammalian organisms, on the contrary,

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lived but a short time. Those receiving bovine type bacilli survived from 10 to 13 days and those receiving human tubercle bacilli lived from 17 to 19 days. From Table I it may be seen that mucin did not effect an appreciable alteration of the survival time in any instance. Later a discussion will be given of the clinical course of the disease, together with weight and temperature curves and pathological findings.

TABLE I
Survival Time and Pathological Findings in First Group of Guinea Pigs Inoculated Intracerebrally

Animal No.	Strain of tubercle bacillus	Suspended in	Survival days	Extent of tuberculosis in				No. of tubercle bacilli in		
				Meninges and brain	Spleen	Liver	Lung	Meninges and brain	Spleen	Lung
4451*	Avian TS	Saline	11	++ ±†						
4450	" "	Mucin	37	+++ ±	+	0	±	+++	+	0
4452	" "	Saline	37	++++	+	0	0	+++	+	0
4449	" "	Mucin	49	++++	+++	+++	±	+++ ±	+	0
4453	Bovine 36	"	10	+++ ±	±	±	0	+++ ±	+	+
4454	" "	"	10	+++ ±	0	±	0	+++	0	0
4455	" "	Saline	11	++++	0	0	0	+++	0	0
4456	" "	"	13	++++	0	0	0	+++	0	0
4457	Human (Fox)	Mucin	17	++++	++	+	0	+++ ±	0	0
4459	" "	Saline	17	++++	++	+	0	+++ ±	++	0
4458	" "	Mucin	19	++++ ±	+	+	0	+++	++	0
4460	" "	Saline	19	++++	++	+	±	+++	+	0

* These are serial numbers of animals used in this laboratory over a period of years.
† ± is used to designate half the value of +. When used alone it indicates a minimal quantity.

This experiment confirms the observation of previous workers that intracerebral inoculation of tubercle bacilli induces an acute and rapidly fatal disease. In addition, it is shown that avian tubercle bacilli introduced by this route cause death in guinea pigs, but the length of survival varies greatly. With mammalian organisms, animals inoculated with the same dose of the same strain survive approximately the same number of days.

The question at once arose as to whether this regularity of the time of death would maintain with any dose of mammalian organisms; and what the effect of smaller dosage would be on the incubation period as well as survival time. The next experiment elucidates this point.

Second Experiment.—Purpose: To determine the effect of diminishing the intracerebral dose of tubercle bacilli on incubation period and survival time.

This experiment was done in two parts. In the first part, forty-eight guinea pigs were divided into twelve groups of four animals each. Each group included one animal heavier, one lighter, and two very near the average weight which was 505 gm. Three strains of tubercle bacilli, including two human type (Fox and O'Donnell) and one bovine (strain 36) were used, four groups of animals receiving any one strain. The volume of the inoculum was constant: 0.1 cc., given intracerebrally as before; but four different doses of each organism were given. The doses were 10^{-1} , 10^{-3} , 10^{-5} , and 10^{-6} mg. and represent respectively, according to Baldwin, Petroff, and Gardner (15) and Calmette (12), 5,000,000, 50,000, 500, and 50 bacteria. By this simple titration it was hoped that an end-point of dosage would be reached at which some animals would survive. Following inoculation, those receiving bovine organisms were kept in a separate room from those receiving human type strains. Otherwise maintenance was identical. The temperature of each animal was taken by rectum daily in the forenoon beginning on the 13th day after inoculation. Each animal was weighed once weekly and again at death.

In the second part of the experiment, twenty-four animals were used and again divided into groups of four each. The average weight was 420 gm. and all animals weighed within 60 gm. of this figure. The total weight of each group was as nearly as possible the same. In this instance the H-37 human strain was used and the organisms were suspended first in saline solution, then the final dilution was made with serum. The inoculating dose was therefore contained in 10 per cent physiological saline and 90 per cent sterile serum, either normal horse serum or normal rabbit serum. The doses of tubercle bacilli were 10^{-1} mg. (5,000,000), 10^{-3} mg. (50,000), and 10^{-5} mg. (500). Each dose in normal horse serum was administered to one group of four animals; and each dose contained in normal rabbit serum was given to one group of four. The temperature of each animal receiving 10^{-3} mg. was taken daily. Weights were determined on all animals twice weekly.

Results. Temperature.—It was found that the temperature was the best index of the condition of the animal at any period of the disease. Contrary to the observation of Shope and Lewis (10) who observed

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no temperature reaction in their animals, we observed a quite characteristic temperature curve as follows. On the 1st day or 2 after inoculation, the temperature was usually elevated to 103.0° or 104.0°F . Thereafter the temperature was normal for a few days, or nearly so. Following this there was a rise in temperature, a little greater each day, for about 4 days to a peak of 104.5 – 106.0°F . No symptoms were usually noted until about the time of the highest fever. The peak of fever was maintained for a day or so, then there was a gradual decline in temperature with concomitant increasing severity of symptoms. The decline in temperature was sometimes abrupt; at other

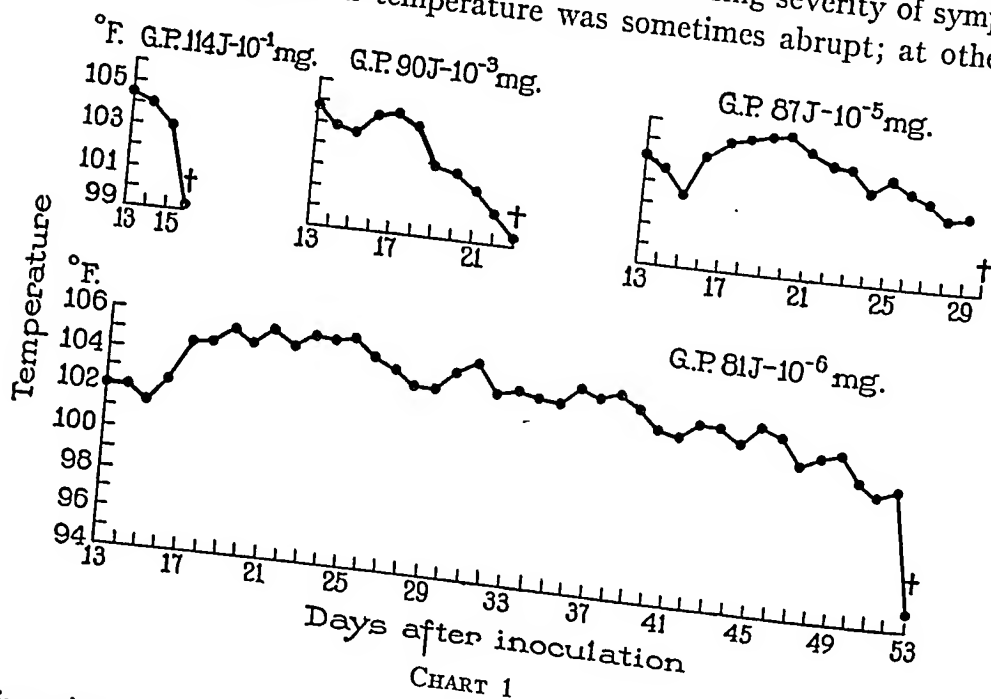


CHART 1

times it lasted 4 or 5 days. But in most instances the temperature during the final 24 hours of life was subnormal, and sometimes markedly so, temperatures as low as 87.0°F .² having been observed. With diminishing doses of organisms, all phases of the temperature curve were elongated except the original post-inoculation rise. It was stated above that the temperature curve was the most reliable index because

² In order to obtain such low temperature readings, it was necessary to shake the mercury down as far as possible, insert the thermometer, then determine the reading (below the graduated scale) with calipers.

of the fact that there was invariably a rise in temperature before any other evidence of disease developed. Chart 1 shows the temperature curves of four animals, each receiving different doses of the O'Donnell strain of human tubercle bacilli. As will be seen (Chart 1), these records began on the 13th day of the disease. The first three animals were individuals whose survival time was nearest the average of the group. The fourth animal, guinea pig 81 J, survived longer than the average of the group but illustrates well the temperature curve observed in animals receiving a minimal dose of organisms. Chart 1 also shows that three of the four animals exhibited a terminal

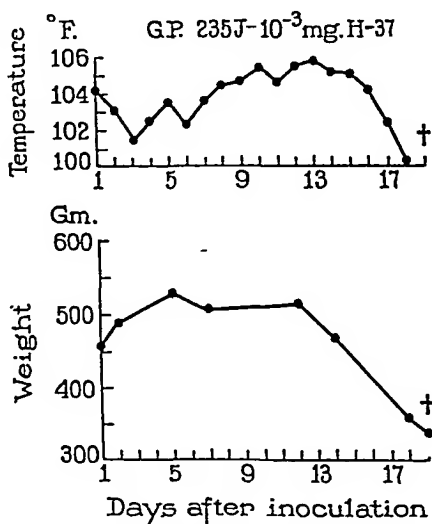


CHART 2

subnormal temperature, while the final reading on the fourth animal was about normal. On Chart 2 may be seen the temperature and weight records of guinea pig 235 J, which received 10⁻³ mg. of the H-37 strain. The initial post-inoculation rise in temperature is shown. Also note that the temperature was elevated for several days before the animal began to lose weight.

Loss of Weight.—There was frequently a moderate loss of weight during the first 2 or 3 days after inoculation. This was almost invariably regained, however, and most of the animals gained a little weight, or at least maintained their pre-inoculation weight until

about the time the peak of fever was reached. Thereafter there was a sharp loss of weight which was progressive until death occurred. The average loss of weight computed from the pre-inoculation weight and that at death in 65 animals with all doses and all strains of organisms was 32.36 per cent of the body weight. Of these 65 animals, only two failed to show a loss in weight, and these were animals which received an inoculating dose of 0.000001 mg. and survived about 1 month each. In some animals emaciation was extreme, due in part to the fact that they were paralyzed and unable to eat during the last hours of life. The fact that loss of weight did not begin until about the time of maximum fever is illustrated on Chart 2 which shows the weight and temperature curves of the same animal. It will be noted that this animal had temperatures exceeding 104°F. on 5 successive days before weight loss was initiated.

Clinical Course of the Disease.—The first noticeable symptom was listlessness. The animals became less active in voluntary movements, and were infrequently heard to make a sound. Appetite at this time was usually well maintained. Next the gait became abnormal; it was of a waddling type, unsteady and hesitating, and marked weakness of the hind quarters was evident. The coat became markedly ruffed in most instances. Paralysis, usually spastic, often but not invariably ensued. This was manifest in one or both hind legs but rarely in the fore legs, so that the animals were capable of locomotion by means of the fore legs. An occasional animal exhibited circular movements to one or the other side. Convulsions were noted in perhaps half the animals but occurred only at a late stage in the disease. Preceding death by 12 to 24 hours there was usually stupor, the animal lying on its side but able to make incoordinated movements when aroused. The respirations were usually somewhat accelerated and deep at this time, the temperature subnormal, and convulsions or rigors were noted.

Pathological Findings.—If the animal had lived as long as 14 days after inoculation, the scalp wound was healed and the skin clip usually missing. Even the trephine opening in the skull was frequently healed. Some, but not all animals showed a tiny yellow nodule in the meninges opposite the point of inoculation. There was marked hyperemia of the meninges and brain, most marked at the base of the brain and over the cerebellum. Macroscopic tubercles were not usually seen, and when they occurred were extremely small and opalescent. The meninges were usually moderately thickened.

The cervical lymph nodes were invariably involved, although tubercles were not visible to the unaided eye in about one-third of the cases. Other lymph nodes were not ordinarily involved.

The spleens were not enlarged but usually showed a few pin-point tubercles. Microscopically the spleen was tuberculous in almost every instance. In Experiments 1 and 2, only four spleens failed to show microscopic tubercles and three of these were from animals (in Experiment 1) which died in 10, 10, and 11 days respectively. Those animals which survived longest usually exhibited the more extensive tuberculosis of the spleen. But it must be emphasized that involvement of the spleen did not cause splenomegaly, as is the case in animals inoculated by other routes than the intracerebral.

Metastatic lesions were next most frequently seen in the liver, but were for the most part microscopic, small, interlobular aggregations of epithelioid cells without giant cells. Of the animals in Experiments 1 and 2, ten only showed absence of tubercles in the livers. Lesions in this organ were more constant and more extensive after human than after bovine tubercle bacilli.

Other organs than the above were not regularly involved.

Of the adrenals from 54 animals, only four showed lesions. These were always microscopic. Only four of the same 54 animals showed pulmonary tuberculosis and two of these were the same animals which showed lesions in the adrenals. Pulmonary lesions were minimal in extent and always microscopic. In thirty-eight animals we obtained two to four sections of lungs, a total of 92 sections, and studied likewise the tracheal lymph nodes from the same animals. Whereas only four of the thirty-eight lungs (92 sections) showed tuberculosis, the tracheal lymph nodes of twenty-four of the same animals were tuberculous. The lung sections from one animal showed lesions when the lymph node was negative (probably from the opposite side). In the other three animals showing pulmonary tubercles the lymph node was also involved; so that there were twenty-one instances in which the tracheal node was tuberculous and the lung not. From these twenty-one animals we examined forty-eight sections of lungs, —not less than two from any animal. This result might seem to indicate some lymphatic connection between the cervical nodes (always

tuberculous in these experiments) and the tracheal nodes. The only other explanation for such a result would appear to be that these lungs contained small tuberculous foci not included in the sections.

Sections of the kidney from each animal failed to show tubercles. The reproductive organs and intestines were likewise invariably negative.

Microscopically, the sections of the brains showed extensive generalized meningitis involving principally the pia at the base of the brain, over the medulla and cerebellum, and extending into the brain substance along the blood vessels. Lesions over the vertex were less extensive than elsewhere; but the right hemisphere was usually as extensively involved as the left. Formed tubercles were the exception, not the rule. Rather the lesions consisted of a diffuse reaction of granulocytes and large mononuclear cells (monocytes and epithelioid cells, Fig. 11), with smaller numbers of lymphocytes, particularly in the older lesions. Necrosis occurred in small foci which were relatively remote from the small arteries (Fig. 8); that is to say, the tuberculous tissue around these vessels rarely showed necrosis. But everywhere were polymorphonuclear cells showing degenerative change. Fig. 1 shows a characteristic field in the meninges over the occipital lobe. It may be seen that the dura is intact, while the underlying structures are massively involved in the tuberculous process. Fig. 2 shows the perivascular infiltration with mononuclears and granulocytes in the brain substance adjacent to the lateral ventricle. Fig. 3 shows similar perivascular infiltration at the base of the frontal lobe.

The lesions involving the cerebellum were formed by direct extension from tuberculosis in the meninges, or from lesions along the blood vessels. Cerebellar lesions were very marked in some animals. Characteristic foci of pathology in the cerebellum are shown in Figs. 4 and 5. Giant cells were very infrequently seen in the lesions.

The possible means by which the infection spreads from the brain to remote organs were: by the lymphatic extension and by way of the blood stream. That spread occurred through the lymphatics is certain from the fact that the cervical lymph nodes were invariably tuberculous. That metastatic lesions may also have arisen by direct invasion of the blood stream is indicated by the fact that occasionally

tuberculous lesions were seen which perforated to the lumen of meningeal veins. Fig. 6 shows a vein at the base of the brain cut longitudinally, with its lumen occluded at one point by tuberculous tissue. Some of the cells in this tissue contained acid-fast bacilli; in fact some cells containing acid-fast bacilli were seen adjacent to the blood in the open portion of the vessel. Fig. 7 shows a vein in the ventricle similarly involved.

Sections of the brains stained with hematoxylin and anilin-fuchsin showed many acid-fast bacilli in the lesions. In animals receiving the largest doses, the bacilli were very numerous, as may be seen in Fig. 8, from an animal injected with 0.15 mg. bovine strain 36. In general the animals receiving bovine organisms tended to show somewhat greater numbers of organisms in the lesions than those receiving human type strains. As an example, Fig. 8, showing many bacilli, may be compared with Fig. 9, showing fewer, the latter from an animal receiving 0.15 mg. of the Fox human strain. This may have been due to the fact that there were greater numbers of organisms per unit weight in the suspensions of bovine organisms inoculated, or to more rapid multiplication of the bovine organisms *in vivo*. That multiplication of organisms *in vivo* did take place may be seen from the number of organisms in Fig. 10, from an animal inoculated with 10^{-6} mg. (approximately 50 bacteria). Many fields in this section showed as many tubercle bacilli as were inoculated. Many of the bacilli were intracellular, while others were obviously extracellular. In which situation multiplication occurred can only be surmised. However, short chains of two or three bacilli lying end-to-end, which we interpret as evidence of multiplication, occurred both intra- and extracellularly. Fig. 11 shows intracellular bacilli in short chains. This photograph represents an area in the meninges over the cerebellum.

Not only were tubercle bacilli found without difficulty in the lesions of the brain, but they were also numerous in metastatic lesions. In the spleens of animals inoculated intracerebrally, bacilli were often more numerous than it has been our experience to find them in animals inoculated subcutaneously.

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Survival Time.—Perhaps the most important features of the disease produced by intracerebral inoculation of tubercle bacilli were the acuity of the disease and the uniformity of survival time in comparable

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animals. First, as to the acuity of the infection, it may be stated that, of all animals included in Experiments 1 and 2, only three fatalities occurred after the 5th week and two of these were on the 36th day. The experiments were therefore completed in a relatively short time. But more important still is the fact that all animals receiving a given dose of a given strain of bacilli survived approximately the same number of days. For example, of the animals receiving the bovine

TABLE II
Survival Time in Days of Guinea Pigs Inoculated with Various Doses of Mammalian Tubercle Bacilli Suspended in Saline Solution

Dose	Strain of tubercle bacilli		
	Human O'Donnell	Human Fox	Bovine 36
	Survival days	Survival days	Survival days
10 ⁻¹ Average	15, 16, 16, 21* 17	19, 19, 20, 28 21.5	11, 13, 13, 15 13
10 ⁻³ Average	21, 21, 23, 25 22.5	22, 23, 25, 32 25.5	18, 19, 19, 22 19.5
10 ⁻⁵ Average	25, 27, 30, 36 29.5	26, 32, 33, S 30.3+	22, 24, 29, 32 26.75
10 ⁻⁶ Average	23, 53, S 38+	27, 31, S, S 29+	30, 31, 36, S 32.3+

One animal inoculated with 10⁻⁶ mg. O'Donnell strain died of intercurrent disease and is not included in these data.

* Each figure represents one animal. S indicates that the animal survived.

strain 36 (Table II), those inoculated with 0.1 mg. died in 11 to 15 days, those with 0.001 mg. died in 18 to 22 days, those with 0.00001 mg. died in 22 to 32 days, and of those with 0.000001 mg., three died between the 30th and 36th days, while one survived. The average survival times were: 13 days after inoculation of 0.1 mg., 19.5 days after 0.001 mg., 26.75 days after 0.00001 mg., and 32.3+ days after 0.000001 mg. Comparable results were obtained with the Fox and O'Donnell (human) strains, as seen in Table II, and with

the H-37 human strain, as seen in Table III. The fact that 0.000001 mg. is near the end-point of virulence for at least three strains of tubercle bacilli may also be seen in Table II, since with this dose one or two animals in each group survived. The prolongation of the incubation period, and of the disease itself, brought about by diminishing doses, is such that a one hundredfold dilution of dosage allowed an increase of 4 to 7 days in the survival time.

Third Experiment.—Purpose: (a) to compare the effects of virulent and attenuated tubercle bacilli when introduced intracerebrally; and

TABLE III

Survival Time in Days of Guinea Pigs Inoculated with Various Doses of H-37 Human Tubercle Bacilli Suspended in Normal Horse or Normal Rabbit Serum

H-37	Serum	
	Normal horse	Normal rabbit
	Survival	Survival
mg.	days	days
10 ⁻¹	11, 17, 19, 24	10, 15, 15, 17
Average	17.75	14.75
10 ⁻³	18, 19, 19, 22	18, 18, 23*
Average	19.5	19.6
10 ⁻⁵	24, 26, 27, 31	21, 26, 27, 31
Average	27.0	26.25

* One animal died with pneumonia and is excluded.

(b) to compare the effects of viable and heat-killed tubercle bacilli of the same virulent strain.

Three male guinea pigs, average weight 543 gm., were each inoculated intracerebrally with 0.1 mg. of H-37 human tubercle bacilli (virulent). Three male guinea pigs, average weight 530 gm., were each inoculated on the same day, intracerebrally with 0.1 mg. bovine tubercle bacilli strain B-1 (attenuated).

At a later date two male guinea pigs, weighing 360 and 400 gm., each were inoculated intracerebrally with 0.1 mg. of living tubercle bacilli, strain H-37. At the same time, two other male guinea pigs, weighing 380 and 390 gm., were inoculated intracerebrally with 0.1 mg. of the same suspension of H-37 but which had been heated 30 minutes in a boiling water bath.

Results.—One of the three animals inoculated with bovine B-1 strain died from general peritonitis following accidental perforation of the intestine with a thermometer. This animal will be excluded from further consideration. A second animal became paralyzed 59 days after inoculation and died on the 64th day. Sections of the brain showed a single tubercle at the base of the brain with marked evidence of healing. No tubercle bacilli could be found in the lesion. There was a small group of giant cells in the cerebellum at one point—also without bacilli. The cervical lymph nodes were extensively tuberculous but all other organs were normal. The other animal receiving 0.1 mg. of bovine B-1 showed no marked temperature reaction, gained weight, and is alive and healthy 117 days after inoculation. The three animals receiving H-37 at the same time died on the 15th, 17th, and 24th days with typical tuberculous meningitis.

The two animals receiving heat-killed H-37 remained well and gained weight, whereas those receiving the same dose of viable bacilli died on the 26th and 31st days³ respectively.

This experiment demonstrates that the clinical course and fatal issue are brought about by the properties of virulence associated with the inoculated bacilli, since the result is quite different when viable attenuated bacilli or heat-killed virulent organisms are used.

DISCUSSION

In any problem selected for experimental investigation it is highly desirable that the methods used and results obtained be capable of standardization. In research in the field of infectious disease, the result of any inoculation of animals which is least subject to experimental error is survival or death from effects of the inoculation. The value of the result is likewise greatly enhanced if the fatal result occurs at precisely or approximately the same time in individuals receiving the same inoculum and the same post-inoculation therapeutics. Such an optimum result has by no means been obtainable heretofore in tuberculosis research; the method described in this paper, however, goes far toward standardization of experimental tuberculosis.

³ The culture with which these animals were inoculated was of a line which is slightly attenuated, thus the longer survival than in previous animals receiving the H-37 strain.

The technique of operation in order to make the inoculation intracerebrally is simple and the cost of instruments negligible. The hazard of the operation to the experimental animal is small. The time consumed by the operation and inoculation is little or no greater than that necessary for an intravenous inoculation. The results obtained by this method have many advantages. First, the acuity of the disease is such that experiments are terminated rather quickly with consequent considerable saving in time, and in cost of animal maintenance. A fatal result is obtainable with sufficiently small doses of organisms that the method gives promise of service as a test of prophylactic or therapeutic measures. But finally and most important of all perhaps, is the fact that the infection induced by intracerebral inoculation of tubercle bacilli in guinea pigs is standardizable, and therefore permits the performance of quantitative titration experiments. The results of such experiments, expressed in terms of survival time, should be readily applicable to statistical methods of analysis.

Certain possible applications of the method herein outlined are at once apparent. It seems not unreasonable to believe that it might be of value in testing the virulence of tubercle bacilli, for tests of the efficacy of prophylactic or therapeutic measures, and in testing antisera to determine whether antibody against tubercle bacilli or antibody against any antigens derived from them have protective properties. Certain of the above possibilities are under investigation in this laboratory at present, and it may be stated that the method is of value in testing the pathogenic properties of *Mycobacteria*.

Recently Neiman and Woolpert (16) have employed the intracerebral route for inoculating tubercle bacilli into fetal and new-born guinea pigs. In comparing their results with ours, it is apparent that fetal or new-born animals are not more susceptible to tuberculosis by this route of inoculation than are older animals; indeed, the reverse may be true. It is interesting to note, however, that they also obtained an acute and fatal disease.

From our results it is evident that a dose of 0.000001 mg. (moist weight, or about 50 organisms) is near the end-point of virulence for at least three strains of mammalian tubercle bacilli, since a certain number of animals which receive this quantity of bacilli survive. Of

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Recently Neiman and Woolpert (16) have employed the intracerebral route for inoculating tubercle bacilli into fetal and new-born guinea pigs. In comparing their results with ours, it is apparent that fetal or new-born animals are not more susceptible to tuberculosis by this route of inoculation than are older animals; indeed, the reverse may be true. It is interesting to note, however, that they also obtained an acute and fatal disease.

From our results it is evident that a dose of 0.000001 mg. (moist weight, or about 50 organisms) is near the end-point of virulence for at least three strains of mammalian tubercle bacilli, since a certain number of animals which receive this quantity of bacilli survive. Of

five such animals which recovered, only one exhibited a temperature reaction at any time during the disease. This animal had temperatures exceeding 104.0°F. on 4 successive days, the highest being 105.2°. None of the five exhibited symptoms. Four of the five were skin tested with old tuberculin 5 months after inoculation; two showed negative and two showed positive reactions, the individual which had shown a temperature rise giving a negative reaction. Since it is not possible by the methods at our disposal to make perfect suspensions of tubercle bacilli, it is probable that the number of organisms injected into these five animals was extremely small. With improved methods of preparing fine suspensions of the bacteria, such as that proposed by Corper and Cohn (17), it is possible that the end-point of virulence might be extended and that even greater uniformity of longevity might be obtained.

SUMMARY

Intracerebral inoculation of tubercle bacilli into normal guinea pigs induces acute meningoencephalitis with minor metastatic lesions. The disease is fatal in a relatively short time and is characterized by a rather typical succession of symptoms and a fairly characteristic temperature curve. The disease is produced by very small numbers of bacilli; and under standard conditions, survival time is so uniform as to make possible quantitative or titration experiments. Certain possible applications of the method are discussed.

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EXPLANATION OF PLATES

PLATE 42

FIG. 1. Section of the left hemisphere of the brain of guinea pig R 4613 which received 0.1 mg. of the O'Donnell (human) strain of tubercle bacilli intracerebrally and died on the 16th day thereafter. Photograph shows the meninges over the occipital lobe with dura intact and extensive tuberculous exudate in the pia-arachnoid space. Note the lack of necrosis. Hematoxylin and eosin. $\times 105$.

FIG. 2. Section of the right hemisphere of the brain of guinea pig R 4455; inoculated with 0.15 mg. bovine tubercle bacilli and died on the 11th day. The photograph shows an area adjacent to the lateral ventricle in which there was marked perivascular infiltration of the brain substance with tuberculous tissue consisting of monocytes and polymorphonuclear leucocytes. This illustrates the mode of local spread of the lesions. Hematoxylin and eosin. $\times 105$.

FIG. 3. Section of the right hemisphere of the brain of guinea pig R 4457 which received 0.15 mg. of the Fox (human) strain intracerebrally and died on the 17th day. Photograph at the base of the frontal lobe showing tuberculous meningitis and perivascular lesions in the brain substance. Hematoxylin and eosin. $\times 70$.

FIG. 4. Section of the left cerebellar hemisphere of guinea pig R 4587 which received 0.000001 mg. of the O'Donnell (human) strain intracerebrally and died on the 53rd day. The photograph shows a tuberculous lesion (arrow) in the granular layer of the cerebellar cortex. Many bacilli were seen in this lesion. Hematoxylin and eosin. $\times 105$.

PLATE 43

FIG. 5. Section of the left cerebellar hemisphere of guinea pig R 4594 which received 0.000001 mg. of the human strain O'Donnell intracerebrally and died on the 25th day following. Photograph shows extensive tuberculous meningitis with perivascular extensions into the cerebellar cortex. Hematoxylin and eosin. $\times 70$.

FIG. 6. Section of the left hemisphere of the brain of guinea pig R 4458 which received 0.15 mg. of the Fox human strain and died on the 19th day. The photo-

graph shows a vein in the meninges at the base of the brain, cut longitudinally and occluded by tuberculous tissue. The cells marked E are the endothelial cells. Tubercle bacilli in the area are indicated by arrows. Hematoxylin and anilin-fuchsin. $\times 450$.

FIG. 7. Section of the left hemisphere of the brain of guinea pig R 4612 which was inoculated intracerebrally with 0.1 mg. of the O'Donnell strain (human) and died on the 21st day following. The photograph shows a vein in the ventricle cut longitudinally with the lumen filled with tuberculous tissue. Vascular endothelial cells are indicated by E. A tubercle bacillus is clearly shown at the point indicated by the arrow. Hematoxylin and anilin-fuchsin. $\times 450$.

PLATE 44

FIG. 8. Section of the left brain of guinea pig R 4455 which received intracerebrally 0.15 mg. of bovine tubercle bacilli, strain 36, and died on the 11th day. Photograph of the meningeal exudate at the base of the brain showing numerous tubercle bacilli, indicated by the arrows. Hematoxylin, anilin-fuchsin, and light green. $\times 1050$.

FIG. 9. Section of the right brain of guinea pig R 4457 which received 0.15 mg. of the human strain Fox and died on the 17th day. Meningeal tuberculous exudate composed chiefly of mononuclear cells at the base of the brain, showing numerous tubercle bacilli, indicated by the arrows. Hematoxylin, anilin-fuchsin, and light green. $\times 1050$.

FIG. 10. Section of the right brain of guinea pig R 4614 which received intracerebrally 0.000001 mg. of the bovine strain 36 and died on the 31st day. The number of bacilli present, indicated by arrows, shows that multiplication of the organisms must have occurred. Hematoxylin, anilin-fuchsin, and light green. $\times 1050$.

FIG. 11. Section of the right brain of guinea pig R 4589 which received 0.001 mg. of the human strain O'Donnell and died on the 21st day. Note presence of bacilli within cells, especially in the multinucleated cells (M). Bacilli lying end-to-end, as indicated by the arrows, are believed to indicate multiplication of the bacteria. Hematoxylin, anilin-fuchsin, and light green. $\times 1000$.



graph shows a vein in the meninges at the base of the brain, cut longitudinally and occluded by tuberculous tissue. The cells marked E are the endothelial cells. Tubercle bacilli in the area are indicated by arrows. Hematoxylin and anilin-fuchsin. $\times 450$.

FIG. 7. Section of the left hemisphere of the brain of guinea pig R 4612 which was inoculated intracerebrally with 0.1 mg. of the O'Donnell strain (human) and died on the 21st day following. The photograph shows a vein in the ventricle cut longitudinally with the lumen filled with tuberculous tissue. Vascular endothelial cells are indicated by E. A tubercle bacillus is clearly shown at the point indicated by the arrow. Hematoxylin and anilin-fuchsin. $\times 450$.

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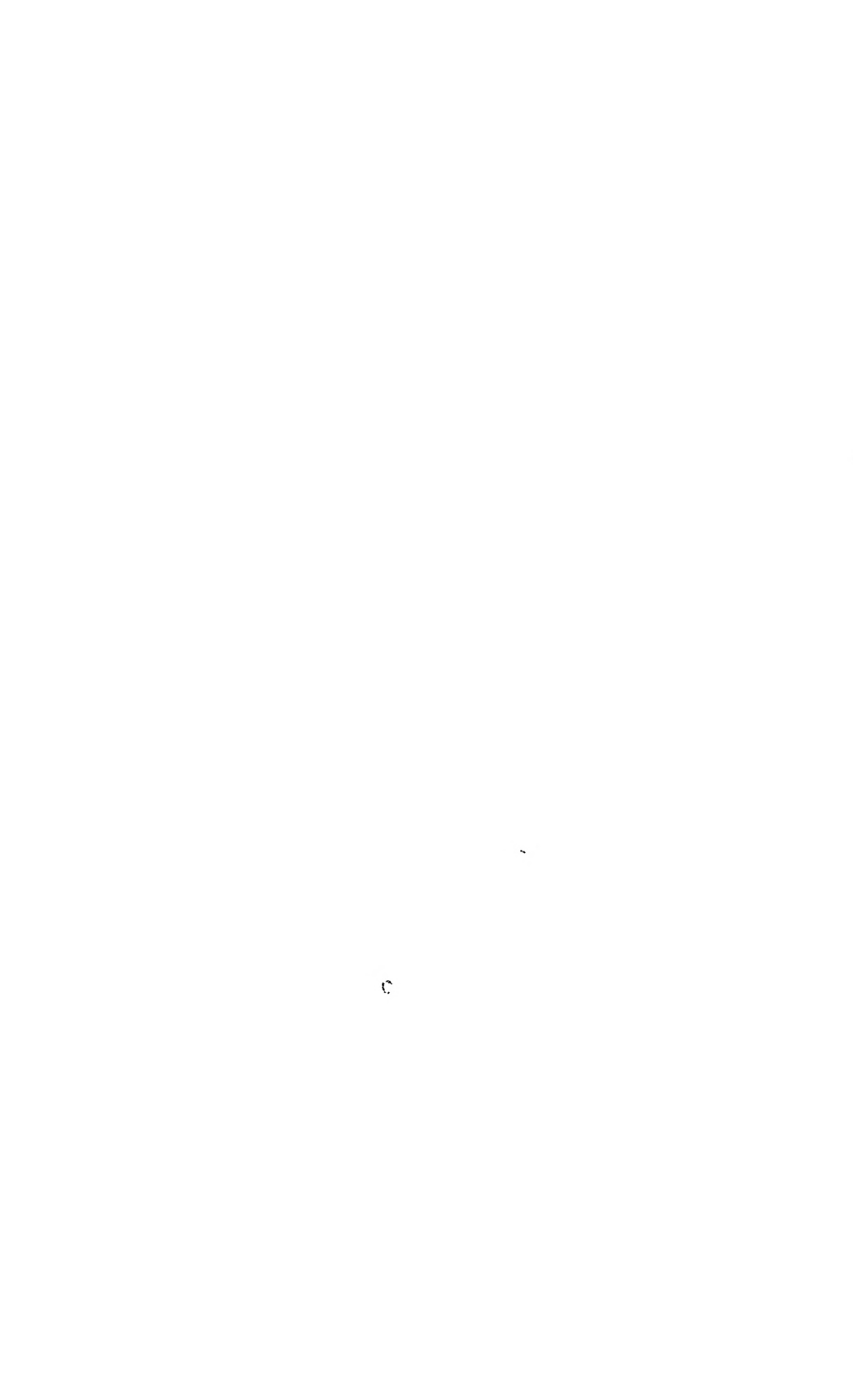
Photographed by Louis Schmidt

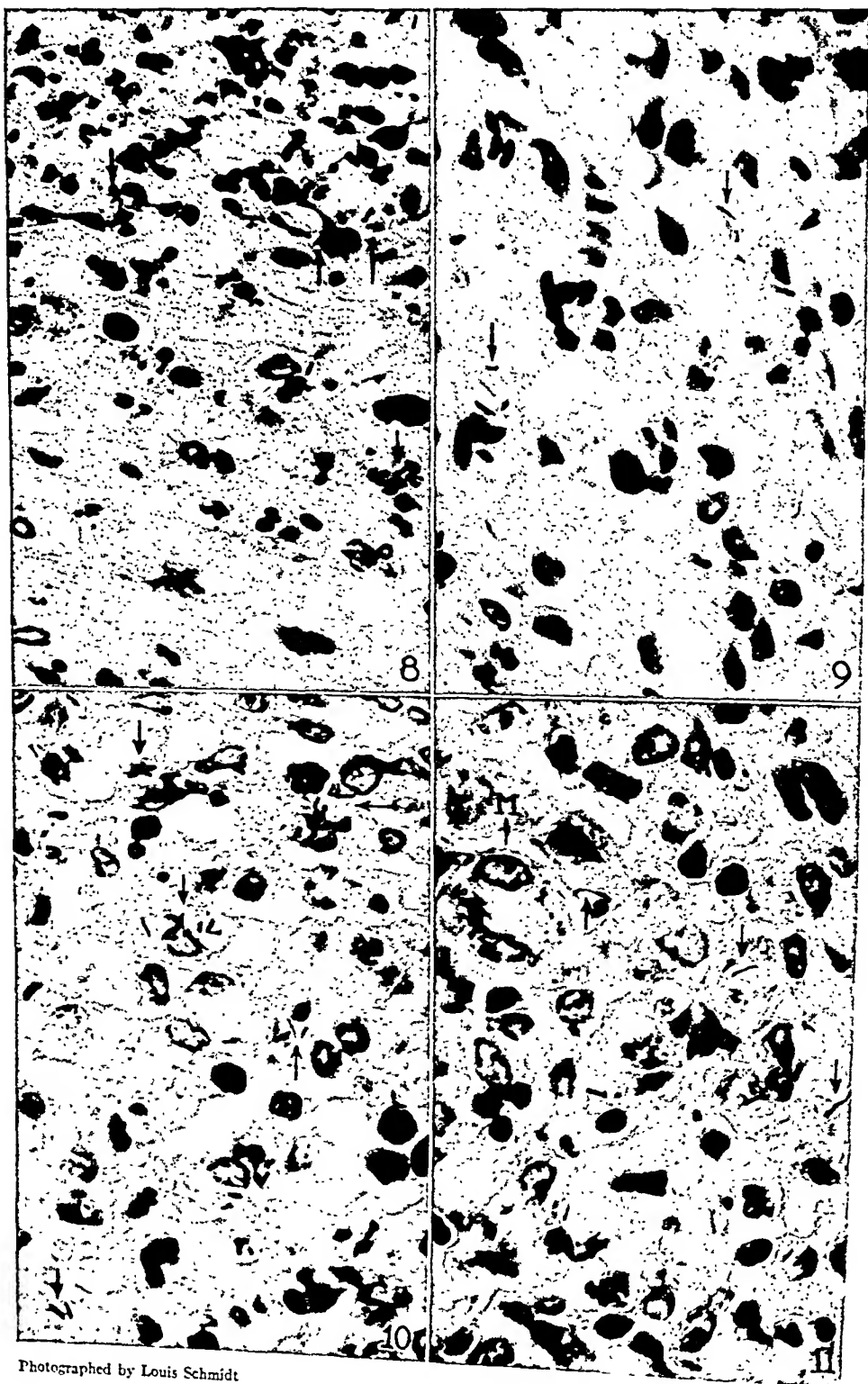
(Smithburn: Longevity in experimental tuberculosis)



Photographed by Louis Schmidt

(Smithburn: Longevity in experimental tuberculosis)





Photographed by Louis Schmidt

(Smithburn: Longevity in experimental tuberculosis)

THE SUSCEPTIBILITY OF SWINE TO THE VIRUS OF HUMAN INFLUENZA*

BY RICHARD E. SHOPE, M.D., AND THOMAS FRANCIS, JR., M.D.

*(From the Department of Animal and Plant Pathology, The Rockefeller Institute
for Medical Research, Princeton, N. J., and the Hospital of The Rockefeller
Institute for Medical Research, New York)*

PLATES 45 TO 48

(Received for publication, June 26, 1936)

The similarity in the pathogenic properties of human and swine influenza virus for ferrets led Elkeles (1) to attempt the transmission of the human agent to swine. He found that very young pigs (2 to 6 weeks old), developed a mild illness when the virus from man was given intranasally under light ether narcosis. At autopsy these animals sometimes showed scattered dark red bronchopneumonic areas of consolidation in the upper lobes of the lung. When cultures of either swine or human influenza bacilli were added to the virus at the time of its administration, the swine developed a more severe illness. The clinical picture was characterized by a low grade fever, apathy, loss of appetite, and sometimes cough. At autopsy varying degrees of bronchopneumonia were encountered. Virus, pathogenic for ferrets, could be recovered from the pneumonic lungs. It thus appeared that Elkeles had produced a disease somewhat resembling swine influenza by the administration to young pigs of human influenza virus mixed with influenza bacilli of either human or swine origin. This observation made more credible the theory that swine influenza may have arisen as the result of the infection of swine in 1918 from human sources (2-4).

The question of the pathogenicity of human influenza virus for swine was of such importance that it seemed to warrant further investigation. The present paper reports our experience in the transmission of human influenza virus to swine.

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more days in most cases. It tended to be diphasic, frequently being lower on the 2nd than on either the 1st or 3rd day. The animals were depressed, their appetites diminished, and they lay listlessly in their pens. The extreme prostration seen often in swine influenza was not observed in any of the swine infected with human virus and *H. influenzae suis*. Symptoms referable to pulmonary involvement were present but less marked than in swine influenza; respiration was accelerated and the animals exhibited the peculiar type of diaphragmatic breathing best described as "thumping." None, however, appeared to be in particular respiratory distress. 2 animals kept under observation for 2 weeks made uneventful recoveries.

The remaining 9 of the 11 swine which had shown an illness greater than that caused by the virus alone were killed either by stunning and bleeding or by chloroforming on the 3rd or 4th day after infection. Influenza virus was demonstrated in the lungs of all by mouse inoculation and *H. influenzae suis* was isolated, usually in pure culture, from either the pneumonic lung or bronchial exudate of each animal. The respiratory tract lesions encountered at autopsy were similar in character to but less extensive than those seen in swine influenza. The trachea contained a scant to moderately abundant, thick, tenacious, glassy mucous exudate. A similar but more copious exudate was present in the bronchi and, in the smaller bronchi and bronchioles of pneumonic lobes of the lung, it completely filled the lumen. The pneumonia was of the same character as that seen in swine influenza but much less extensive. Seldom were more than two lobes involved, whereas in swine influenza an involvement of five lobes is the rule, and not uncommonly portions of all seven lobes may be pneumonic. The right cardiac lobe was most frequently affected, next the azygos lobe, and after that either the right apical or upper portion of the right diaphragmatic lobe. Lobes on the left side were seldom observed to be involved. The affected areas of lung were slightly depressed when compared with uninvolved lung and the line of demarcation between normal and pathological lung was sharp. The scattered areas of lobular atelectasis which gave a checker-board appearance to the lungs of animals infected with virus alone were replaced by a confluent pneumonia in which large numbers of adjacent lobules participated. Frequently the entire right cardiac lobe, or most of the azygos lobe, or the whole base of the right apical lobe was consolidated. The involved lung was a purplish red in color, felt firm and leathery, did not crepitate, and, when grasped with forceps, was friable in contrast to its usual rubber-like consistency. The cut surface was moist and the small bronchi which protruded exuded a thick glassy white mucous exudate. A cloudy serous fluid was yielded by the cut lung surface itself. Photographs of the lung of a pig infected with human influenza virus and *H. influenzae suis* are shown in Figs. 5 and 6.

Histologically sections of pneumonic lung cut in such a way as to include small bronchi and terminal bronchioles exhibited the following features: The cilia lining the smaller bronchi were either entirely gone or badly matted together. The bronchial epithelium was fragmented, in places partially desquamated, and,

scattered between the cells, were leucocytes singly or in clumps. The lumina of the bronchi were filled with a polymorphonuclear leucocytic exudate and the bronchial walls outside the epithelium were densely infiltrated with mononuclear cells (Fig. 7). The alveolar walls were thickened and infiltrated with mononuclear cells as were also the interlobular septa. The alveoli themselves contained red blood cells, leucocytes, and coagulated plasma (Fig. 8). Leucocytes were most abundant in the alveoli opening directly into the terminal bronchioles. Dilated capillaries in the alveolar walls were packed with red blood cells and widened lymph channels in the interlobular septa were filled with coagulated lymph and small numbers of cells.

From the above descriptions it is apparent that the pathological process caused in the hog lung by infection with a mixture of human influenza virus and *H. influenzae suis* differs qualitatively as well as quantitatively from that produced by infection with either the swine or the human influenza virus alone, while it differs only quantitatively from that seen in swine influenza (infection with a mixture of swine influenza virus and *H. influenzae suis* (10)).

As stated earlier, only 11 of the 16 swine to which human influenza virus and *H. influenzae suis* were administered developed an illness more severe than that caused by the virus alone. The remaining 5 pigs exhibited symptoms indistinguishable from those encountered in swine infected with the virus alone. At autopsy the lungs of these animals showed lesions characteristic of those caused by the virus alone and cultures of the trachea, bronchi, and lungs failed to reveal the presence of *H. influenzae suis*. Influenza virus was, however, demonstrated in the lung lesions by mouse inoculation. Thus in these 5 swine, inoculated in the usual fashion with human influenza virus and *H. influenzae suis*, the virus but not the bacterium had become established in the respiratory tract. Instances of this nature have never been encountered in swine inoculated with swine influenza virus and *H. influenzae suis*.

Attempted Transmission of Human Influenza Virus by Contact in Swine

One of the characteristic features of swine influenza, as seen in the field, is its extreme contagiousness. This high degree of communicability may also be demonstrated in animals experimentally infected with swine influenza virus and *H. influenzae suis*. In a series of experiments that is now large, the swine influenza virus has rarely failed to transfer from sick to normal swine by pen contact, although under certain conditions (12) the accompanying bacterium may fail to do so.

In four experiments to test the communicability of human influenza virus, normal swine were placed in the same pens with pigs inoculated intranasally with either human influenza virus alone or human virus mixed with *H. influenzae suis*. None of the 4 exposed animals developed clinical evidence of illness. The respiratory tract of one, killed on the 4th day following exposure, appeared normal at autopsy and its turbinates and lung, tested by mouse inoculation, were found to be free of virus. The remaining 3 pigs were kept under observation for 3 weeks. Their sera were then tested for ability to neutralize human influenza virus in mice. The serum of one animal neutralized the virus completely while that of the other 2 contained no demonstrable antibodies for human influenza virus. Thus, virus had been transferred by pen contact to only 1 of the 4 animals exposed. In this single case the disease was too mild to be recognized clinically although it did result in the establishment of specific virus-neutralizing antibodies. A comparison of this small group of human virus experiments with similar contact experiments in which swine virus was employed leads to the conclusion that human influenza virus is much less communicable in swine than swine influenza virus.

Cross-Immunity in Swine Convalescent from Infection with Swine or Human Influenza Virus

In cross-immunity experiments thus far conducted it has been found that swine recovered from swine influenza are immune to infection with a mixture of human influenza virus and *H. influenzae suis*. Experiments to test the immunity to swine influenza conferred by a preliminary infection with human influenza virus have indicated that the development of cross-immunity in this direction may, to some extent at least, be influenced by whether the initial infection was with human virus alone or with this virus and *H. influenzae suis*. The results of these experiments, which are still in progress, will be reported later. Sera of swine recovered from infection with swine influenza neutralize the swine influenza virus but exert little or no neutralizing effect on the human virus, while sera of swine recovered from infection with human influenza virus neutralize only the human agent (13).

Identification of Human Influenza Virus after Serial Passage in Swine

It was important in the present experiments to establish that the influenza virus transferred in series through swine was actually a human type agent and not a swine influenza virus accidentally introduced during passage. All possible precautions as to isolation of animals were practiced throughout the period of investigation and

experiments with swine influenza virus were discontinued so far as possible while the human virus was under study. The possibility of cross-infection, though remote, was nevertheless recognized and for this reason at the termination of the present study with the human virus in swine the following two experiments were conducted. First, known anti-swine and anti-human influenza virus immune sera were tested for their ability to neutralize the virus recovered from the 5th and last serial passage swine. The virus was neutralized by anti-human but not by anti-swine virus sera. Second, convalescent sera from three 5th passage swine were tested for their ability to neutralize human and swine influenza virus; they neutralized only human virus. It is clear from these results that the virus, recovered after serial passage in swine, was the human influenza virus entirely unaltered immunologically from that with which the studies were begun.

DISCUSSION

The present experiments confirm Elkeles' observation that swine are susceptible to human influenza virus. Contrary to Elkeles' observations, however, it was not necessary to use baby pigs or to anesthetize the animals in order to induce infections. The lack of agreement may possibly be due to differences in the natural susceptibility of the swine used in our experiments and the Dutch pigs employed by Elkeles. In this respect, it is of interest that *Ferkelgrippe*, an enzootic pneumonia of baby pigs prevalent in Germany, is believed by Köbe (14) and Waldmann (15) to have a complex etiology similar to that of swine influenza and is transmissible only to young pigs. It is possible, from consideration of Elkeles' work, that, as in the case of *Ferkelgrippe*, only the very young of European breeds of swine may react to infection with human influenza virus.

When the disease produced in swine by the combined action of human influenza virus and *H. influenzae suis* is compared with swine influenza, it is apparent that the two are similar qualitatively but different quantitatively. The increased severity of the pneumonia produced by the swine virus and bacterium in comparison with that produced by the human virus and bacterium seems to constitute a significant difference between the strains of human and swine influenza viruses studied. An explanation of this difference in the two viruses

is not evident but it may be that human virus possesses less power than swine virus to prepare an extensive area of lung for the invasion of *H. influenzae suis*. A second possible explanation may be that the present human influenza virus is inherently less capable of acting synergistically with a second agent than is swine influenza virus. The failure of *H. influenzae suis* to establish itself in the respiratory tracts of 5 of 16 swine to which it was given in admixture with human virus, as contrasted with its invariable establishment in swine when administered in combination with swine influenza virus, would support this second possibility.

Elkeles' experiments, and those presented here, have shown that a virus from cases of influenza in man is capable of infecting swine when administered intranasally, that the pathogenic properties of this virus are usually enhanced by the presence of *H. influenzae suis*, and that the resulting pneumonia is qualitatively similar to that seen in swine influenza. However, it seems unlikely, in view of its low communicability, that this recent strain of the human influenza virus could establish itself in swine and progress as the cause of any widespread or serious epizootic disease. In this respect, it is of interest that two strains of swine influenza virus (Iowa, 1934, and Ohio, 1935), recovered from hogs since the present human strain was known to be prevalent, are serologically the same as swine influenza virus strain 15 (Iowa, 1930) and thus serologically different (2, 13, 16, 17) from human influenza virus of the WS or PR 8 type.

As mentioned earlier, it has been suggested that swine were originally infected with influenza from man in 1918 and that the swine virus is the surviving prototype of the virus prevalent at that time in the human population (2-4). If it could be assumed, for the sake of the present discussion, that swine influenza etiologically is a replica of the human pandemic disease and that man and swine react alike to infection with virus and bacterium, then the differences, discussed above, in the pathogenicity for swine of the viruses of swine and recent human influenza might reflect differences between severe pandemic influenza as it occurred in 1918 and the recent milder interpandemic form from which both the PR 8 and WS strains of virus were obtained. The swine influenza virus, highly communicable and capable of causing an extensive pneumonia when acting synergistically with a second

organism, would be expected to result in a disease that varied both epidemiologically and clinically from that caused by the recent human influenza virus, an agent less capable of acting synergistically with a second organism and less communicable.

SUMMARY

Swine inoculated intranasally with human influenza virus alone develop an ill defined, mild, and usually afebrile illness of short duration. At postmortem the anterior lobes of the lungs of such animals contain scant, scattered areas of lobular atelectasis. Transmission of the virus for 5 serial passages through two groups of swine failed noticeably to enhance its pathogenicity for this species. The disease produced in swine by infection with human influenza virus alone is indistinguishable clinically and pathologically from that caused by infection with swine influenza virus alone. Transmission of human influenza virus from swine to swine by contact succeeded in only one of four attempts.

Swine inoculated intranasally with a mixture of human influenza virus and *H. influenzae suis* usually develop a febrile, depressing illness similar to mild swine influenza. The pneumonia encountered in such animals at autopsy is similar to but less extensive than that seen in swine influenza. In some animals *H. influenzae suis* fails to become established and the disease then seen is identical with that caused by human influenza virus alone.

The human influenza virus recovered after 5 serial transfers in swine was immunologically the same as that with which the experiments were begun.

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EXPLANATION OF PLATES

PLATE 45

FIG. 1. Dorsal aspect of lung of swine infected with PR 8 strain human influenza virus alone. There is a scattered, patchy, lobular atelectasis of the right cardiac lobe and the upper portion of the right diaphragmatic lobe. Animal chloroformed on 3rd day after infection.

FIG. 2. Ventral aspect of same lung.

PLATE 46

FIG. 3. Section of lung of a swine infected with PR 8 strain human influenza virus alone showing folded, thickened alveolar walls infiltrated with mononuclear cells. The small bronchi are cuffed by accumulations of round cells. Animal chloroformed on 3rd day after infection. Phloxine-methylene blue. $\times 68$.

FIG. 4. Higher power of above section to show round cell infiltration of the alveolar walls in an area of atelectasis. $\times 262$.

PLATE 47

FIG. 5. Dorsal aspect of lung of swine infected with mixture of PR 8 strain human influenza virus and *H. influenzae suis*. There is an atelectatic pneumonia of the right cardiac lobe. Animal chloroformed on 3rd day after infection.

FIG. 6. Ventral aspect of same lung. The pneumonia involves all of the right cardiac lobe and lobular areas of the azygos and upper portion of the right diaphragmatic lobes.

PLATE 48

FIG. 7. Section of a small bronchus in lung of a swine infected with mixture of PR 8 strain human influenza virus and *H. influenzae suis* showing leucocytic bronchial exudate, fragmented and vacuolated bronchial epithelium denuded of cilia, and round cell infiltration of the submucosa. Leucocytes have invaded

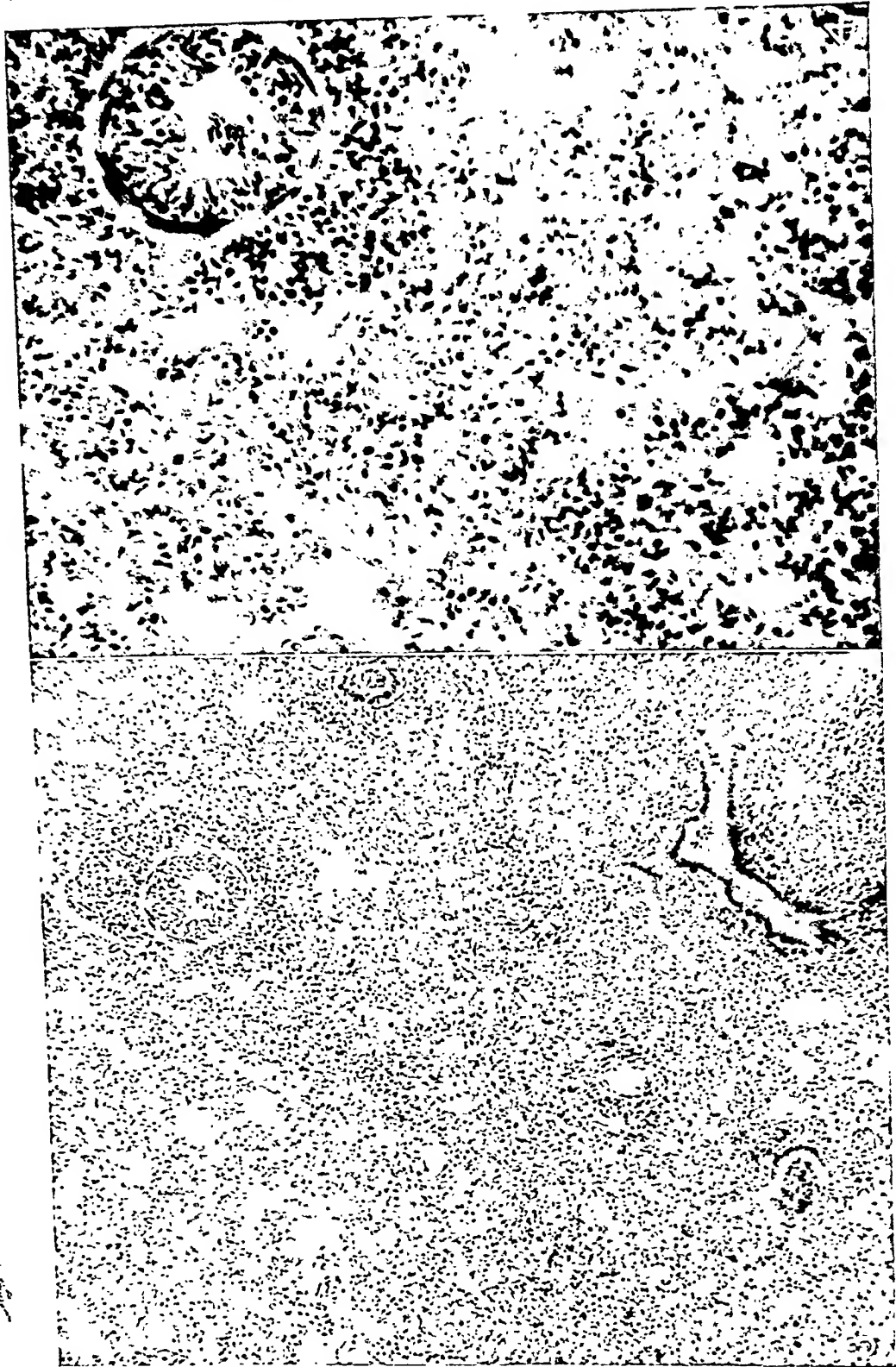
he mucosa. Animal chloroformed on 3rd day after infection. Phloxine-methylene blue. $\times 245$.

FIG. 8. Section of lung of a swine infected with mixture of PR 8 strain human influenza virus and *H. influenzae suis*. The small bronchi contain a dense polymorphonuclear leucocytic exudate and are cuffed by round cells. The alveolar walls are thickened and infiltrated with round cells and some of the alveoli contain accumulations of polymorphonuclear leucocytes. Animal chloroformed on 3rd day after infection. Phloxine-methylene blue. $\times 56$.

2



1



Photographed by J. A. Carlile

(Shope and Francis: Susceptibility of swine to human influenza)

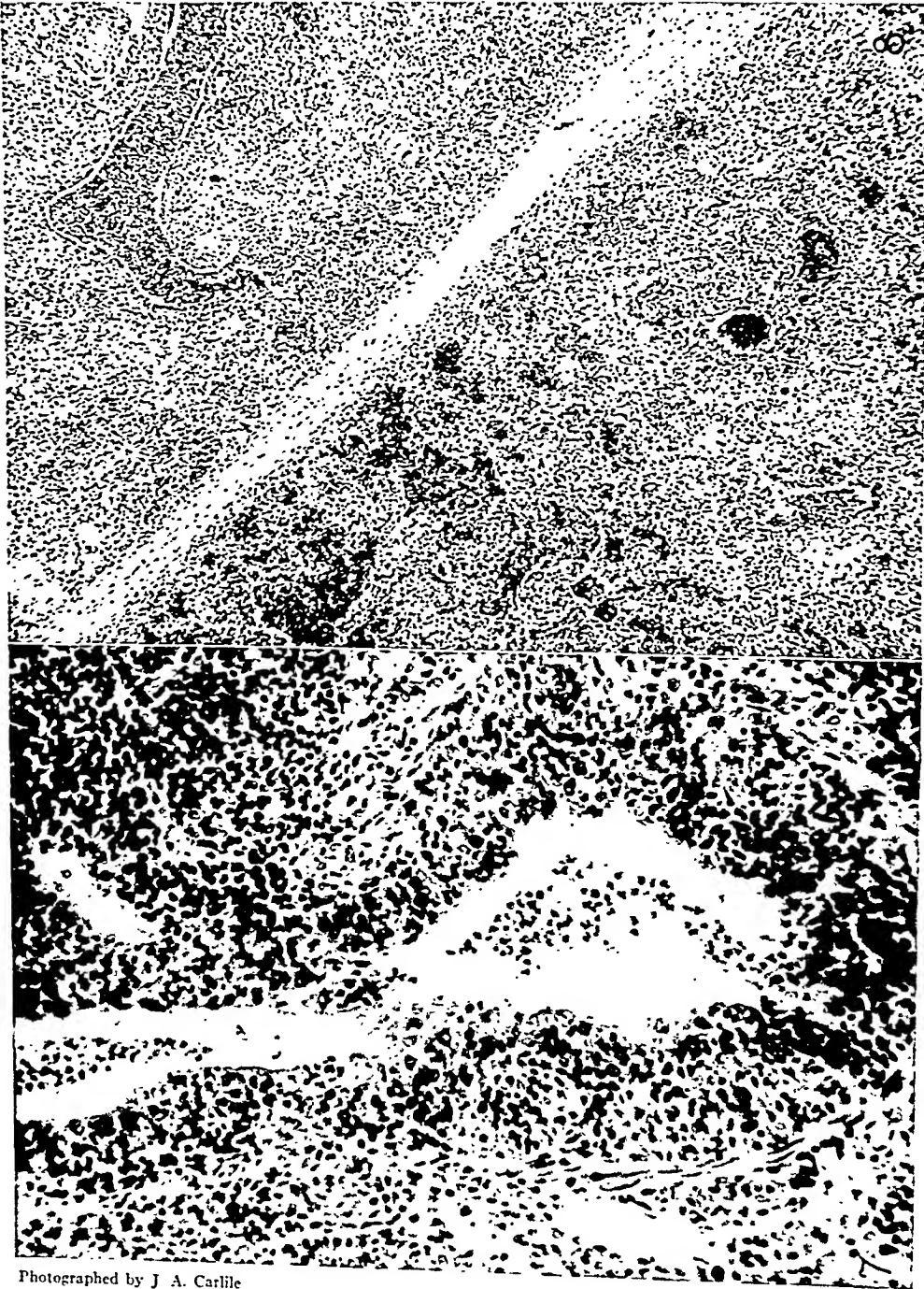
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Photographed by J. A. Carlile

5

(Shope and Francis: Susceptibility of swine to human influenza)



Photographed by J. A. Carlile

(Shope and Francis: Susceptibility of swine to human influenza)

STUDIES ON THE SOLUBLE PRECIPITABLE SUBSTANCES OF VACCINIA

I. THE DISSOCIATION IN VITRO OF SOLUBLE PRECIPITABLE SUB- STANCES FROM ELEMENTARY BODIES OF VACCINIA

BY JAMES CRAIGIE, M.B., CH.B., AND F. O. WISHART, M.D.

(From the Connaught Laboratories and School of Hygiene, University of Toronto,
Toronto, Canada)

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Soluble substances which precipitate in the presence of vaccinia immune serum are found in extracts of fresh dermal vaccine, and may be separated from the elementary bodies of vaccinia by means of centrifugation and filtration (Craigie, 1932; Parker and Rivers, 1935). Craigie (1932) carried out cross-absorption tests which showed a serological relationship between these soluble precipitable substances and the elementary bodies, and he therefore concluded that the former were specific products of the elementary bodies. Comparatively large amounts of these substances are present in solution in fresh suspensions of vaccine pulp and it must be inferred that they exist in this state *in vivo* or are very rapidly liberated from the virus when the vaccine pulp is suspended in dilute buffer or saline solution.

It has been found that the elementary bodies contain at least two agglutinogens (L and S) which differ markedly in their stability, particularly to heat. The L agglutinin is thermolabile at 56°C. while the S agglutinin is stable at 95°C. (Craigie and Wishart, 1934). In view of this antigenic complexity of the elementary bodies, the soluble precipitable substances have been reinvestigated in regard to their serological relationships to the elementary bodies.

In addition to the soluble precipitable substances which are distinguished by their occurrence in suspensions of fresh dermal vaccine, precipitable substances have been recovered from suspensions of washed elementary bodies and these form the subject of the present paper. In order to distinguish them from the precipitable substances

found in fresh vaccine pulp preparations (p.p. substances), they will be termed dissociated antigens. Where this distinction is not possible, the term precipitable substance will be used.

EXPERIMENTAL

The Dissociation of Antigen from Elementary Bodies

The method of preparing suspensions of elementary bodies for use in agglutination tests, which was described by Craigie and Wishart (1934), included three successive washings of the elementary bodies in the angle centrifuge. The object of this treatment was to dilute the p.p. substances well beyond the point at which they might conceivably interfere with the agglutination reaction, and failure of the final washings (third supernatant) thus obtained to give a precipitin reaction with antivaccinia serum was regarded as evidence that this had been accomplished. Suspensions of elementary bodies prepared in this way were stored in dilute buffer solution under ether in the cold room until required. In subsequent investigation of the L and S agglutinogens, it was observed that S precipitable substance was present in solution in suspensions of elementary bodies which had been heated to inactivate the L antigen. However, control tests showed that heating was not entirely responsible for the liberation of S antigen, for most suspensions prior to heating were found to contain free precipitable substances which remained in the supernatant fluid when the elementary bodies were centrifuged out. Elementary body suspensions of varying age were therefore centrifuged and the supernatants subjected to the precipitin test. Most of these supernatant fluids gave positive reactions in dilutions ranging from 1 in 2.5 to 1 in 80. Portions of thrice washed suspensions were therefore centrifuged at intervals after preparation and the supernatants examined. While in these earlier experiments no precipitable substances were found in solution immediately after preparation of the suspensions, they were detected on the following day and increased in amount for some days subsequently. More recently, by the use of pure L and pure S precipitin sera instead of L and S agglutinating sera, this liberation of precipitable substances from elementary bodies has been reinvestigated. The preparation of the precipitin sera and the reasons for referring the L and S antibodies to the corresponding precipitinogens

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in preference to agglutinogens will be discussed in subsequent papers in this series. It is sufficient at this point to state that the L type of serum is specific for precipitinogen thermolabile at 56–60°C., while the S type is specific for a heat-stable precipitinogen.

Nine suspensions of elementary bodies were prepared for a quantitative study of the dissociation of antigen from them. Titrations were made (a) of the p.p. substances in the pulp supernatant on centrifuging out the elementary bodies, (b) of the amounts of precipitable substances in two immediately consecutive supernatant fluids resulting from the washing of the elementary bodies, and (c) of the precipitable substances in the dilute buffer solution in which the elementary bodies were suspended for 4 to 7 days. The same L and S sera were used throughout for titration of the precipitable substances. The titrations were carried out by means of the incubation of a series of dilutions of the solutions with a constant dilution of serum for 18 hours at 50°C.

Pyrex tubes, internal dimensions 4 mm. by 12 mm., and 120 mm. in length, were used for centrifugation of the suspensions. After each centrifugation the supernatant fluid was decanted and the tubes inverted on filter paper to drain for a few moments. The possibility of the elementary bodies being affected by slight drying was avoided by omitting any attempt to remove traces of fluid adhering to the walls of the tube. The maximum volume of the fluid remaining in the tube was estimated to be 0.025 cc. Since 2.5 cc. of dilute buffer solution per tube were employed after each deposition to resuspend the elementary bodies, the minimum dilution of free precipitable substance carried over from the pulp suspension ing the amount of precipitable substance was 1 in 100. In calculating the amount of precipitable substance on each washing was 1 in 100. In calculating the amount of precipitable substance carried over from the pulp suspension to the washed elementary body suspension, we have arbitrarily decreased this dilution to 1 in 40 to offset any errors due to volumes larger than 0.025 cc. being retained on draining the tubes. On this basis the amount of precipitable substance to be expected in the suspending fluid of thrice washed elementary bodies would be less than $1/64,000$ of that found in the first supernatant. The ratio of the observed amount of precipitable substance to the calculated amount was determined for the 2nd and 3rd supernatant fluids and for the fluid in which the suspensions were stored in the cold room. The maximum and minimum ratios obtained for the supernatant fluids from the nine elementary body suspensions are given in Table I, while the actual precipitin titres observed in the case of all of the suspensions are shown in Table II.

As Tables I and II show, from 1600 to 12,800 times the calculated amount of both L and S precipitable substances appeared in solution. This result, taken in conjunction with the results of the other experiments, leads to the conclusion that precipitable substances may dissociate from elementary bodies *in vitro* therefore appears to be justified. The figures

TABLE I

The Ratio of the Observed to the Calculated Amounts of Antigen Present in Elementary Body Suspensions

Supernatant*	L antigen		S antigen	
	Minimum ratio	Maximum ratio	Minimum ratio	Maximum ratio
Second	4 to 1	8 to 1	4 to 1	8 to 1
Third	20 to 1	80 to 1	20 to 1	80 to 1
Fourth	1600 to 1	12,800 to 1	1600 to 1	12,800 to 1

All tables in this paper relate to the C.L. lapine strain of virus (Craigie and Wishart, 1934).

* Second and third supernatants—consecutive wash waters of elementary bodies immediately following their separation from fresh dermal vaccine pulp.

Fourth supernatant—obtained after storage of the washed elementary body suspensions for 4 to 7 days in the cold room.

Nine lots of elementary bodies were investigated, but only the minimum and maximum ratios obtained are stated.

TABLE II

Elementary body harvest	Supernatant*	Titre of L antigen	Titre of S antigen	Ratio of observed to calculated antigen	
				L antigen	S antigen
No. 77	1st	1 in 200†	1 in 400†	—	—
	2nd	1 in 20	1 in 40	4 to 1	4 to 1
	3rd	1 in 5	1 in 5	40 to 1	20 to 1
	4th	1 in 10	1 in 10	3200 to 1	1600 to 1
No. 116	1st	1 in 400†	1 in 400†	—	—
	2nd	1 in 40	1 in 40	4 to 1	4 to 1
	3rd	1 in 5	1 in 10	20 to 1	40 to 1
	4th	1 in 40	1 in 80	6400 to 1	12,800 to 1

* First, second, and third supernatants were immediately consecutive washings of the elementary bodies after their separation from fresh dermal vaccine pulp. The fourth supernatant was obtained after the elementary body suspensions had been stored in the cold room.

† Titres of p.p. substances from which the titres of the calculated antigen for subsequent washings were determined.

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Tables I and II also indicate that the process of dissociation was not delayed until after the third washing but occurred to some extent throughout the manipulation of the suspensions.

A considerable variation in the amount of antigen that becomes dissociated in different suspensions of elementary bodies has been observed. Successive lots of suspension have shown considerable differences in the ratio of elementary bodies to amount of antigen dissociated, although these lots were derived from one batch of seed elementary bodies and prepared precisely in the same way. The highest yields of dissociated antigen have given precipitation in dilu-

TABLE III

Exhausted elementary body suspension	Serum dilutions						Precipitin serum
	1 in 20	1 in 40	1 in 80	1 in 160	1 in 320	1 in 640	
No. 349	—	—	—	—	—	—	Normal serum
	+++	+++	+++	++	+	±	L serum
	+++	+++	+++	++	+	±	S serum
No. 350*	+	+	+	+	+	+	Normal serum
	++	++	++	++	++	+	L serum
	++	++	++	++	+	+	S serum
No. 355	—	—	—	—	—	—	Normal serum
	++++	++++	+++	++	±	—	L serum
	++++	+++	+++	+++	+++	±	S serum

Tests incubated for 18 hours at 50°C.

* Suspension 350 partially unstable.

tions up to 1 in 80 while some suspensions have failed to yield any demonstrable antigen. If the first yield is high, further but progressively smaller amounts of antigen are liberated when the elementary bodies are resuspended in dilute buffer solutions. Ultimately dissociation of a demonstrable amount of antigen ceases and the exhausted suspension tends to become unstable. Partial instability, as far as the agglutination test is concerned, can usually be overcome by the removal of the larger clumps of elementary bodies by light centrifugation and the performance of the test in solutions containing 0.3 to 0.05 per cent NaCl and 5 per cent normal rabbit serum. Tests

done in this way (Table III; suspension 350) show that the less unstable bodies in an exhausted suspension retain their agglutinability in L and S sera, but it has not been possible to determine whether the completely unstable bodies retain antigen on their surface.

Exhausted suspensions usually remain highly infective. This is shown in Table IV which relates to a number of suspensions 3 to 7 months old. Of these, suspensions 229, 269, 272, 292, and 309 had

TABLE IV

Elementary body suspension No.	A*			B†		
	Titre of D.A.		Titre of virus‡	Titre of D.A.		Titre of virus‡
	L serum	S serum		L serum	S serum	
224	1 in 40	1 in 40	1 in 10 ⁶	1 in 10	1 in 20	1 in 10 ⁶
229	1 in <5	1 in <5	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁶
269	1 in <5	1 in <5	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁶
272	1 in <5	1 in <5	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁶
292	1 in 10	1 in 20	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁶
309	1 in <5	1 in <5	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁴
311	1 in 10	1 in 40	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁴
319	1 in 20	1 in 40	1 in 10 ⁶	1 in 10	1 in 10	1 in 10 ⁶
326	1 in 10	1 in 20	1 in 10 ⁶	1 in 10	1 in 10	1 in 10 ⁶
330	1 in 40	1 in 40	1 in 10 ⁶	1 in <5	1 in <5	1 in 10 ⁶
360	1 in 40	1 in 40	1 in 10 ⁶	1 in 20	1 in 20	1 in 10 ⁶

* The elementary body suspension was centrifuged and the amount of dissociated antigen (D.A.) in the supernatant was titrated. The elementary bodies were resuspended in dilute buffer and titrated intradermally (0.2 cc. volumes intradermally).

† After 3 weeks in the cold room the suspensions were again centrifuged and dissociated antigen and infectivity titrated.

‡ Infectivity was not titrated in dilutions beyond 1 in 10⁶.

previously been centrifuged to obtain dissociated antigen. The primary object in examining these suspensions was to procure exhausted suspensions of fairly high infectivity and thus no attempt was made to titrate the virus beyond 10⁻⁶. The elementary bodies were titrated for infectivity after they had been centrifuged and resuspended in dilute buffer solution, while the supernatant fluid remaining after deposition of the elementary bodies was titrated for dissociated antigen. After the resuspended elementary bodies had

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remained for a further 3 weeks in the cold room, they were again centrifuged and their infectivity and any further amounts of antigen dissociated during the 3 week period were determined.

As Table IV shows, most of the suspensions were infective in dilutions as high as 10^{-6} . Fresh elementary body suspensions of this strain of virus show an average intradermal titre of 10^{-7} . It is evident, therefore, that a high degree of infectivity remains after further dissociation of antigen is no longer demonstrable. It should be noted that the infectivity of two suspensions (309 and 311) dropped from 10^{-6} to 10^{-4} without any concomitant liberation of antigen.

Microscopic examination has failed to show any consistent changes in suspensions which might be correlated with the dissociation of antigen. On two occasions material of doubtful significance has been fractionated from suspensions. The suspensions were centrifuged without added NaCl and the slightly opalescent supernatants were recentrifuged after addition of NaCl to 0.85 per cent. Clear supernatant fluids were thus obtained which on being tested showed an antigen titre of 1 in 80. A small amount of deposit (estimated at approximately $\frac{1}{10}$ of the volume of the elementary bodies) was obtained on clearing each opalescent supernatant fluid and was found to be serologically inert. Viewed by dark ground illumination with a Zeiss HI60 objective, this deposited material showed masses of particles approximately similar in size to elementary bodies, but of very low luminosity. The nature of these ghost particles has not been determined nor can their presence in the suspension of thrice washed elementary bodies be accounted for. If they were present in the original pulp suspension, it is difficult to explain why they were not removed by the three washings in dilute buffer solution to which the elementary bodies were subjected.

Factors Influencing Dissociation of Soluble Antigen

Seitz filtrates of vaccine pulp suspensions contain in addition to the precipitable substances large amounts of protein, and are therefore unsuitable for precipitin absorption tests. Dissociated antigen preparations present these substances in a comparatively pure state and numerous attempts have consequently been made to obtain greater yields. However, no method has been found which will significantly increase or even decrease the total amount of antigen dissociated from a suspension, although the velocity of dissociation may be altered to a slight extent. Dissociation takes place more rapidly at 37°C . than at cold room temperatures, when a suspension is approaching the state of exhaustion, but a temperature effect is not

so readily demonstrable with fresh suspensions. After a suspension has apparently become exhausted, small amounts of S antigen may be obtained by heating the suspension at temperatures above 56°C. Dissociation of antigen takes place more readily between pH 7.0 and pH 8.0 and is definitely depressed on the acid side of pH 6.0. Unfortunately, the p.p. substances and some of the protein of vaccine pulp may be thrown out of solution if acidity is increased beyond pH 6.0, and consequently the use of an acid buffer solution in the preparation of elementary body suspensions would considerably reduce the amount of these substances which could be removed by washing. The yield of L antigen is reduced at reactions more alkaline than pH 8.0, probably as a result of partial destruction of this labile substance.

Ether appears to accelerate dissociation to a slight extent, particularly above cold room temperature. A small but consistent difference has also been noted between portions of suspensions suspended in distilled water, dilute buffer solution, and 0.85 per cent NaCl solution respectively, the amount of dissociation being approximately halved in saline solution. The addition of normal rabbit serum to suspensions does not appear to appreciably decrease dissociation. Attempts to disrupt elementary bodies and thus obtain solutions containing both L and S antigens have been unsuccessful. The methods used included (a) resuspension in distilled water after exposure to 10 per cent NaCl for 24 to 96 hours at 37°C., (b) repeated freezing and thawing, (c) repeated desiccation, and (d) violent hammering of suspensions frozen with CO₂ snow and acetone in a steel block. No significant amount of either antigen was obtained by any of these methods.

Properties of the Dissociated Antigens

As has been indicated in Tables I and II, antigens are liberated by elementary body suspensions which are precipitable by L or S precipitin sera prepared by the injection into animals of vaccinia precipitable substances. These antigens are also precipitated by L or S agglutinating sera prepared by hyperimmunization of rabbits with elementary body suspensions. The lability of the dissociated L antigen corresponds to that of the L agglutinin. Altered reactivity as shown by impaired precipitability may be induced by heating at a temperature as low as 50°C., and exposure to 56–60°C. results in

complete inactivation. Examples of this thermolability chosen in order to show the extremes encountered are given in Table V. Other experiments have shown that the L antigen on heating at 56°C. first shows impaired precipitability with a reduction of titre, and that a period of 1 hour may be required for complete inactivation. The S antigen, on the other hand, is stable up to 100°C. but autoclaving at

TABLE V
The Thermolability of Dissociated L Antigen

Dissociated antigen	L serum 202* 1 in 60 Antigen dilutions					
	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80	1 in 160
No. 247 Unheated 1 hr. at 50°C. 1 hr. at 53°C. 1 hr. at 56°C. 1 hr. at 60°C.	.	+++	+++	+	±	—
	.	oo	?	—	—	—
	.	—	—	—	—	—
	.	—	—	—	—	—
	.	—	—	—	—	—
No. 266 Unheated 1 hr. at 50°C. 1 hr. at 53°C. 1 hr. at 56°C. 1 hr. at 60°C.	oo	++	++++	++	+	—
	oo	oo	+	?	—	—
	—	—	—	—	—	—
	—	—	—	—	—	—
	—	—	—	—	—	—
No. 268 Unheated 1 hr. at 50°C. 1 hr. at 53°C. 1 hr. at 56°C. 1 hr. at 60°C.	++++	++	+	—	—	—
	oo	++	±	—	—	—
	o	+	±	—	—	—
	(o)	±	—	—	—	—
	—	—	—	—	—	—

oo and o = opalescence only. Plus signs indicate the amount of precipitate; negative signs indicate absence of precipitation and opalescence.

* L serum 202 was obtained by absorption of the S antibody in the original serum with dissociated antigen heated at 70°C. in order to inactivate the L antigen.

pH 7.0 for 15 minutes at 15 pounds reduces its titre to approximately half.

The effects of formalin on the two antigens offer an interesting contrast which is illustrated in Table VI. Sufficient formalin was added to make the concentrations stated in the table, and the preparations were examined after contact with the formalin in the cold room

for intervals of 20 hours and 4 days respectively. The L antigen shows degrees of inactivation approximately proportional to the concentration of formalin, while the titre of the S antigen preparation is not reduced. The latter preparation, however, shows a reduced precipitability in the region of antigen excess which is proportional to the concentration of formalin.

The relationship between the L and S titres of the dissociated antigen is a fairly constant one, the L titre being half or slightly more

TABLE VI

Treatment of dissociated antigen		L serum (246 Abs. S)* 1 in 25 D.A. 246/1 diluted						S serum (243/19) 1 in 20 Heated D.A. 225/1 diluted				
Time	Concentration of formalin	1 in 2.5	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80
	<i>per cent</i>											
20 hrs.	1.0	++	+	±	—	—	—	00	+	+	±	—
	0.5	+++	++	+	±	—	—	+	+	++	±	—
	0.25	+++	+++	++	+	—	—	+++	++	++	±	—
	Nil	+++	+++	+++	+++	++	±	+++	+++	++	±	—
4 days	1.0	—	—	—	—	—	—	00	00	+	±	—
	0.5	++	±	—	—	—	—	00	00	+	±	—
	0.25	+++	++	±	—	—	—	00	+	++	±	—
	Nil	+++	+++	+++	+++	++	±	+++	+++	++	±	—

* 246 Abs. S = serum 246 after removal of S precipitin with dissociated antigen 229/1.

Heated D.A. = dissociated antigen heated for 1 hour at 70°C. prior to addition of formalin.

than half that of the S titre. Unsuccessful attempts have been made to separate the L and S antigens by absorption of one of the antigens with precipitin sera containing only L or S antibody. The equivalent proportions of L or S antigen and serum were determined by means of the Dean and Webb tests (1926), and absorption tests set up accordingly. The resultant precipitates were centrifuged out and the supernatant fluid examined for residual precipitable substance. It was found that the equivalent amount of pure L or S serum removed both antigens while lesser amounts brought about an equal reduction in the titres

TABLE VII
Combined Precipitation of L and S Substance with S Antibody

	L serum 202, 1 in 40, and supernatant diluted						S serum 203, 1 in 30, and supernatant diluted					
	1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64	1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64
Mixture* from which supernatant was obtained												
D.A., 1.6 cc. + S serum, 0.2 cc. + saline 0.2 cc.....	-	-	-	-	-	-	-	-	-	-	-	-
D.A., 1.6 cc. + S serum, 0.1 cc. + saline 0.3 cc.....	+	+	+	+	+	+	±	+	+	+	+	+
D.A., 1.6 cc. + saline, 0.4 cc.....	+	+	+	+	+	+	00	+	+	+	+	+
D.A., 1.6 cc. + typhoid O												

The supernatant fluid was then tested for residual precipitable substances.

* Mixtures held for 4 hours at room temperature and then centrifuged to remove the precipitate formed.

for intervals of 20 hours and 4 days respectively. The L antigen shows degrees of inactivation approximately proportional to the concentration of formalin, while the titre of the S antigen preparation is not reduced. The latter preparation, however, shows a reduced precipitability in the region of antigen excess which is proportional to the concentration of formalin.

The relationship between the L and S titres of the dissociated antigen is a fairly constant one, the L titre being half or slightly more

TABLE VI

Treatment of dissociated antigen		L serum (246 Abs. S)* 1 in 25 D.A. 246/1 diluted						S serum (243/19) 1 in 20 Heated D.A. 225/1 diluted				
Time	Concentration of formalin	1 in 2.5	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80
20 hrs.	<i>per cent</i>											
	1.0	++	+	±	—	—	—	oo	+	+	±	—
	0.5	+++	++	+	±	—	—	+	+	++	±	—
	0.25	+++	+++	++	+	—	—	+++	++	++	±	—
4 days	Nil	+++	+++	+++	+++	++	±	+++	+++	++	±	—
	1.0	—	—	—	—	—	—	oo	oo	+	±	—
	0.5	++	±	—	—	—	—	oo	oo	+	±	—
	0.25	+++	++	±	—	—	—	oo	+	++	±	—
	Nil	+++	+++	+++	+++	++	±	++++	+++	++	±	—

* 246 Abs. S = serum 246 after removal of S precipitin with dissociated antigen 229/1.

Heated D.A. = dissociated antigen heated for 1 hour at 70°C. prior to addition of formalin.

than half that of the S titre. Unsuccessful attempts have been made to separate the L and S antigens by absorption of one of the antigens with precipitin sera containing only L or S antibody. The equivalent proportions of L or S antigen and serum were determined by means of the Dean and Webb tests (1926), and absorption tests set up accordingly. The resultant precipitates were centrifuged out and the supernatant fluid examined for residual precipitable substance. It was found that the equivalent amount of pure L or S serum removed both antigens while lesser amounts brought about an equal reduction in the titres

of both antigens. Control experiments with other precipitin systems failed to reveal any demonstrable adsorption of L or S antigens by heterologous precipitates. Table VII illustrates the removal of L antigen effected by an S serum. This S serum showed no residual L precipitin when the S antibodies were removed by a heated antigen preparation. Table VII also shows the failure of a precipitate produced by an extract of *B. typhosus* and O serum to adsorb the L or S substances.

The LS antigen dissociated from elementary bodies stimulates the production of L and S antibodies. Further reference to this will be made in a subsequent paper but it may be pointed out here that owing to the comparatively low yields of dissociated LS antigen which can be obtained, such preparations are not suitable for the preparation of potent precipitin sera. As stated above, L and S precipitin sera, obtained by hyperimmunization with preparations of the p.p. substances, were employed for the detection and titration of the L and S antigens dissociated from the elementary bodies. The ability of these sera to agglutinate elementary bodies and precipitate the antigens dissociated from them indicates a serological relationship between the p.p. substances and the agglutinogens of the elementary body which will be considered further in a subsequent paper.

DISCUSSION

The observations reported in this paper indicate that specific precipitable substances dissociate *in vitro* from the elementary bodies of vaccinia. These substances are precipitable both by pure L and pure S precipitin sera and correspond in their thermolability or thermostability to the L and S agglutinogens previously described (Craigie and Wishart, 1934). The addition of an equivalent amount of pure L or pure S precipitin serum to solutions of these substances was found to precipitate both the labile and stable substances and these substances may therefore be regarded as components of a complex LS antigen rather than two separate antigens. Furth and Landsteiner (1929) found that sera containing only one antibody precipitated the whole polysaccharide complex of *Salmonella* species and not merely the homologous fraction of the polysaccharide preparation. Burnet (1934) has advanced analogous observations on the antigens

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of Flexner bacilli in criticism of the mosaic hypothesis. The LS antigen differs, however, from the antigen preparations examined by these authors, in that instead of antigens of similar stability it comprises antigens which differ markedly in this respect.

The supernatant fluid obtained from suspensions of washed elementary bodies which have been preserved under ether would appear to contain the LS antigen in a much less impure state than do Seitz filtrates of vaccine pulp. Unfortunately, no means of increasing the dissociation of this substance from elementary bodies has been found, and the yields obtained are insufficient for adequate absorption tests, thus limiting the application of these comparatively pure preparations in the further investigation of the serology of vaccinia.

Craigie (1932) showed by means of cross-absorption tests that the soluble precipitable substances of vaccine pulp (Seitz filterable flocculable substance) were serologically similar to the agglutinogens of the elementary bodies, and from this he concluded that these precipitable substances were specific products of the elementary bodies of vaccinia. On the other hand, the opinion has been expressed by some that the substances involved in precipitin and complement fixation reactions with certain viruses are specific products of infection and not derived from the virus substance (*vide* Sabin, 1935). As far as extensive observations go, the antigens associated with the agglutination, precipitin, and complement fixation reactions of vaccinia and variola are specifically related to these virus infections. Since infection with these viruses is a necessary preliminary to the appearance of these antigens and the formation of corresponding antibodies, it must be conceded that the antigens are specific products of the virus irrespective of their site and mode of production. The question at issue would thus appear to be the question of whether the antigens are elaborated within the virus and are subsequently liberated *in vivo* or *in vitro*, or whether they are formed outside the virus particle by the action of substances, secreted by the virus, on some constituents of the infected cell. Perhaps the failure of viruses to multiply in the absence of living cells may find an explanation, particularly in the case of the small viruses, in certain stages of the conversion of host substances into virus substance taking place outside of and not within the limits of the virus particle.

The mere dissociation of LS antigen from washed elementary bodies does not provide an answer to this question of intrinsic or extrinsic formation of the antigens of vaccinia for, as Sabin (1935) has pointed out, it might be possible for virus to adsorb specific soluble substances produced by the infective process and thus become agglutinable. According to this view the dissociation of LS antigen *in vitro* would be interpreted as a reversal of an *in vivo* adsorption. Indeed, certain observations noted in this paper might appear to support this view that the LS antigen is an extrinsic, not an intrinsic, product of the virus. Vaccinia elementary bodies retain a high degree of infectivity after dissociation of LS antigen has ceased and subsequent treatment fails to liberate any further demonstrable amount of antigen. However, the ultimate state of extreme instability attained by suspensions still highly infective precludes tests for residual LS agglutinin. Another point is that elementary body suspensions dissociate amounts of LS antigen which are small compared with the amount found in solution in a suspension of fresh vaccine pulp. Our data indicate that a given quantity of fresh vaccine pulp will yield 20 to 50 times as much LS antigen as the total amount of elementary bodies recovered from this amount of pulp will dissociate *in vitro*.

The view that the agglutinogens of the elementary bodies of vaccinia are in reality adsorbed antigens of extrinsic origin involves, however, the assumption that the highly resistant elementary bodies present in repeatedly washed and aged suspensions represent the only form in which vaccine virus may exist. There is very definite evidence that the elementary bodies represent the virus of vaccinia since ability to initiate the vaccinia infection is resident in these bodies and the infective process is accompanied by a great increase in their number. Like crude virus suspensions, the elementary bodies retain their infectivity in the presence of glycerol, ether, or phenol. Ether has been extensively used in the preparation and preservation of elementary bodies, and suspensions kept in dilute buffer solutions saturated with this reagent have been found to retain a remarkably constant infectivity over many months. The survival of infectivity under such conditions indicates a highly resistant, resting phase which contrasts with the rapidity of the proliferation of the virus and of the attendant pathological changes when these resistant bodies are brought into contact with susceptible cells.

It should be pointed out that the method which we have employed to obtain suspensions of elementary bodies (Craigie and Wishart, 1934) may possibly result in a selection of virus particles and that the resistant elementary bodies so obtained may not be strictly representative of all the virus particles present in the original pulp. The most important point about the method in this connection is that the pulp is not triturated. Trituration of the pulp does not significantly increase the total amount of washed elementary bodies recovered, but does introduce technical difficulties in obtaining a pure suspension, since it results in the appearance of minute particles of varying size, presumably produced by cell disruption, which are difficult to separate from the elementary bodies. Presumably, therefore, the elementary bodies obtained by this method are not to any significant extent derived from cells in which infection has proceeded to cell necrosis. The elementary bodies thus obtained retain their infectivity under various adverse conditions for prolonged periods and this implies a very low ebb of metabolic activity. The rapidity of the infective process and the degree of virus proliferation which ensues when susceptible tissue is inoculated with resistant elementary bodies, indicate on the other hand a high degree of vegetative activity *in vivo*. Two forms of vaccine virus may thus be postulated on the basis of metabolic activity, one a resting, highly resistant form represented by the particles in a prepared suspension of elementary bodies, the other the actively proliferating, presumably intracellular form which by its pathogenic activity produces changes in the infected tissue. It is not suggested that there is necessarily any morphological difference between these functionally different forms. It might be expected that the actively vegetative form of the virus would lack the qualities of resistance *in vitro* exhibited by the resistant elementary bodies and that transitional forms of intermediate resistance would also occur.

If the LS antigen is produced by the virus it must be elaborated by the vegetative and not by the resistant form. No evidence is available, however, which would clearly indicate whether all the LS antigen found in solution in fresh pulp suspensions is present mainly in this state *in vivo*, and the possibility of rapid lysis of nonresistant virus particles when the pulp is suspended in saline or dilute buffer should be borne in mind. If virus particles showing various degrees

The mere dissociation of LS antigen from washed elementary bodies does not provide an answer to this question of intrinsic or extrinsic formation of the antigens of vaccinia for, as Sabin (1935) has pointed out, it might be possible for virus to adsorb specific soluble substances produced by the infective process and thus become agglutinable. According to this view the dissociation of LS antigen *in vitro* would be interpreted as a reversal of an *in vivo* adsorption. Indeed, certain observations noted in this paper might appear to support this view that the LS antigen is an extrinsic, not an intrinsic, product of the virus. Vaccinia elementary bodies retain a high degree of infectivity after dissociation of LS antigen has ceased and subsequent treatment fails to liberate any further demonstrable amount of antigen. However, the ultimate state of extreme instability attained by suspensions still highly infective precludes tests for residual LS agglutinin. Another point is that elementary body suspensions dissociate amounts of LS antigen which are small compared with the amount found in solution in a suspension of fresh vaccine pulp. Our data indicate that a given quantity of fresh vaccine pulp will yield 20 to 50 times as much LS antigen as the total amount of elementary bodies recovered from this amount of pulp will dissociate *in vitro*.

The view that the agglutinogens of the elementary bodies of vaccinia are in reality adsorbed antigens of extrinsic origin involves, however, the assumption that the highly resistant elementary bodies present in repeatedly washed and aged suspensions represent the only form in which vaccine virus may exist. There is very definite evidence that the elementary bodies represent the virus of vaccinia since ability to initiate the vaccinia infection is resident in these bodies and the infective process is accompanied by a great increase in their number. Like crude virus suspensions, the elementary bodies retain their infectivity in the presence of glycerol, ether, or phenol. Ether has been extensively used in the preparation and preservation of elementary bodies, and suspensions kept in dilute buffer solutions saturated with this reagent have been found to retain a remarkably constant infectivity over many months. The survival of infectivity under such conditions indicates a highly resistant, resting phase which contrasts with the rapidity of the proliferation of the virus and of the attendant pathological changes when these resistant bodies are brought into contact with susceptible cells.

of transition towards the extremely resistant form occur in vaccine pulp and dissociation of LS antigen is referable to their disintegration, a progressive decrease in the rate of dissociation of antigen similar to that found on washing and storing elementary body suspensions is to be expected. It is not known, however, whether lysis or disintegration of elementary bodies can occur and the dissociation of antigen *in vitro*, which progressively decreases, might be interpreted as elution of LS antigen from the altered limiting membrane of all the resistant virus particles in the suspension.

Interpretation of the observations reported in this paper along these lines provides an alternative to the view that the LS antigen is of extrinsic origin and adsorbed by the elementary bodies. Before the latter view can be accepted some explanation of the apparently specific adsorption of LS antigen by the elementary bodies *in vivo* and the reversal of this process *in vitro* is required. The former view postulates the existence of two functionally different forms of the virus, one of which elaborates the LS antigen and retains some of this antigen on its surface when it changes into the resistant form. Dissociation of this antigen *in vitro* in a suspension of elementary bodies may represent either antigen eluted from the surface of the resistant elementary bodies or liberated by the disintegration of less resistant forms.

CONCLUSIONS

1. Thermolabile and thermostable soluble precipitable substances dissociate *in vitro* from the elementary bodies of vaccinia.
2. These substances are serologically related to the soluble precipitable substances found in suspensions of fresh vaccine pulp.

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STUDIES ON THE SOLUBLE PRECIPITABLE SUBSTANCES OF VACCINIA

II. THE SOLUBLE PRECIPITABLE SUBSTANCES OF DERMAL VACCINE

By JAMES CRAIGIE, M.B., Ch.B., AND F. O. WISHART, M.D.

(From the Connaught Laboratories and School of Hygiene, University of Toronto,
Toronto, Canada)

(Received for publication, June 24, 1936)

In addition to the agglutinable elementary bodies, suspensions of dermal vaccine contain soluble precipitable substances which may be obtained free of virus (Craigie, 1932; Parker and Rivers, 1935). Craigie, by means of cross-absorption tests, showed that the same antibodies which were involved in the agglutination of washed elementary bodies also precipitated these soluble substances. More recently Craigie and Wishart (1934 a) have reported that two distinct agglutinins occur in antivaccinia sera, the corresponding antigens being distinguished by a marked difference in thermostability. One of these antigens, the L agglutinin, is inactivated at 56°C. while the S agglutinin is stable at 95°C. It has also been found that the soluble substances which dissociate *in vitro* from washed elementary bodies contain thermolabile and thermostable components corresponding to the L and S agglutinogens (Craigie and Wishart, 1936). The amount of LS antigen which dissociates *in vitro* from washed elementary bodies is small compared with the amount of soluble precipitable substances (p.p. substances) encountered in fresh vaccine suspensions. It is not known whether the latter substances exist *in vivo* in the state of solution in which they are obtained or whether they are liberated by the virus when vaccine pulp is subjected to manipulation *in vitro*. At present the question of the relationship of these substances to the vegetative form of the virus can be approached only indirectly. One method of approach is by the serological analysis of these substances with reference to the agglutinogens of the elementary bodies. Craigie (1932) found that the elementary bodies

of transition towards the extremely resistant form occur in vaccine pulp and dissociation of LS antigen is referable to their disintegration, a progressive decrease in the rate of dissociation of antigen similar to that found on washing and storing elementary body suspensions is to be expected. It is not known, however, whether lysis or disintegration of elementary bodies can occur and the dissociation of antigen *in vitro*, which progressively decreases, might be interpreted as elution of LS antigen from the altered limiting membrane of all the resistant virus particles in the suspension.

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and the p.p. substances (Seitz filterable flocculable substance) showed a serological relationship. This relationship has been reinvestigated since the elementary bodies have been found to possess both thermolabile and thermostable antigens.

EXPERIMENTAL

The preparations of the soluble precipitable substances of vaccinia used in this investigation were derived from dermal lapine processed to obtain suspensions of elementary bodies. The method used has been described in a previous paper (Craigie and Wishart, 1934 a) which should be consulted for details. When the elementary bodies are deposited by angle centrifugation from the lapine extract the supernatant fluid is passed through a Seitz EK filter. These filtrates contain the soluble precipitable substances in solution, generally in titres ranging from 1 in 400 to 1 in 1600. These crude filtrates have a protein content corresponding to 1 to 2 mg. of nitrogen per cc. or more. Although they give satisfactory precipitation reactions with antivaccinia serum, they are usually unsuited for more elaborate serological tests. If heated for the purpose of inactivating thermolabile substances, crude filtrates tend to develop an undesirable degree of opacity or instability. Even more important, however, is the presence of substances which inhibit precipitation. When a serum is absorbed with untreated filtrate, these inhibiting substances are carried over into the absorbed serum and interfere with its subsequent examination for residual antibodies. In a number of instances, however, satisfactory cross-absorption tests have been carried out with selected filtrates. These tests clearly indicated that substances serologically identical with the L and S components of the LS agglutinin and the dissociated LS antigen were present in these filtrates. In order that these observations on selected filtrates might be confirmed and extended, it was found necessary to devise a method of eliminating the properties of crude filtrates which generally render them unsuitable for absorption tests.

The LS Fraction of Vaccine Filtrates

Wilson Smith (1932) described the preparation of a heat-stable precipitable extract from testicular vaccinia which contained protein

and carbohydrate radicles, while Ch'en (1934), using an elaboration of this method, has obtained a precipitable substance apparently of the nature of a carbohydrate haptene. In repeating and modifying these procedures results were obtained which suggest that the non-antigenic precipitable substances of Smith and Ch'en are components of the antigenic S agglutinin of elementary bodies. Moreover, evidence has been obtained which suggests that the S antigen occurs in combination with the highly labile L antigen (Craigie and Wishart, 1936). It was apparent, therefore, that any method used to purify the precipitable substances prior to serological investigation must avoid disruption of the LS antigen or inactivation of the L component. A preliminary investigation of the precipitation of the precipitable substances by carbon dioxide, chilled alcohol, and by alum yielded unsatisfactory results, but precipitation by an acid buffer solution seemed more promising. A number of buffer systems were tested and the most consistent results were obtained with the HCl-citrate-NaOH system described below. Accordingly, the pH at which maximum precipitation of the LS antigen occurred was determined for various concentrations of electrolyte and subsequently the conditions of pH, electrolyte concentration, and time for maximum solution of the precipitated antigen with minimum solution of precipitated rabbit protein were investigated. A method which has given consistently satisfactory results was thus arrived at. It should be borne in mind that the pH at which the antigen is precipitated from crude filtrates may be influenced by the nature and concentration of the accompanying proteins. The hydrogen ion concentrations stated in the following description of the method are those found to be requisite for 3rd day dermal lapine produced by the C.L. strain of elementary bodies. Before this method is applied to dermal vaccine filtrates from other strains of vaccine virus, particularly strains such as the Armstrong testicular strain, or to extracts of other organs and tissues infected with vaccinia, preliminary tests should be undertaken to determine whether modification of the pH of the acid buffer is necessary.

Preparation of the LS Fraction.—The vaccine filtrate is first subjected to dialysis in cellophane bags. A 1/10 volume of Sørensen's HCl-citrate buffer (Clark, 1928), pH 4.45, is added to the dialysed filtrate and the mixture centrifuged for 30 minutes. The deposit is drained and fluid adhering to the walls of the tube dried

with filter paper. The deposit is then suspended in Sørensen's citrate-NaOH buffer, pH 6.65, the volume used being 1/10 of the final volume of fraction desired. Four times this volume of distilled water is added and after thorough dispersion of the deposit the suspension is centrifuged and the supernatant set aside. The deposit is extracted a second time with the same volumes of buffer (pH 6.65) and distilled water as previously used. The two supernatant fluids thus obtained are pooled and emulsified with ether and after 24 to 48 hours in the cold room are again centrifuged to remove the precipitate which appears. The supernatant fluid thus obtained will be referred to as the LS fraction. Saturation of the fraction with ether provides a convenient method of preventing subsequent bacterial contamination. It should be noted that this method also provides a means of

TABLE I

Nitrogen Content of Lapine Filtrates and Derived LS Fractions*

No. of filtrate and LS fraction	N per cc.	Precipitating titre (L)	N per 100 units of L antigen (approximate)
	mg.		mg.
Filtrate 71	2.39	1 in 400	0.6
LS fraction 71	0.51	1 in 800	0.064
Filtrate 139	1.26	1 in 200	0.63
LS fraction 139	0.12	1 in 400	0.03
Filtrate 175	1.16	1 in 200	0.58
LS fraction 175	0.21	1 in 400	0.053
Filtrate 283	0.882	1 in 400	0.22
LS fraction 283	0.276	1 in 800	0.035

1 unit of LS antigen = amount just yielding perceptible precipitation with optimum amount of L serum in a volume of 0.5 cc.

* Estimated by micro Kjeldahl method.

concentrating the LS antigen by limitation of the volume of buffer used to dissolve the precipitated antigen. The LS fraction thus obtained is, of course, still impure but is much more suitable for serological tests than the original crude filtrate. A considerable amount of protein, however, is soluble at pH 4.45, while further amounts fail to dissolve at pH 6.65 or precipitate when the LS fraction is treated with ether. The proportion of protein to LS antigen is thus reduced and nitrogen estimations indicate that the reduction is an appreciable one.

In Table I the nitrogen content of several crude filtrates and the LS fraction derived from them are shown (a) per cubic centimeter of the material and (b) corrected for the amount of LS antigen present. In

processing, the material was concentrated 2.5 times and the titres of the LS fractions were approximately twice those of the original filtrates.

There is evidence that reprocessing of the LS fraction results in greater purification, but since the inhibitory substances are reduced to a negligible amount by a single treatment, further manipulation has not been found necessary.

Properties of the LS Fraction

Antigenicity.—The LS fraction prepared by the method which has just been described stimulates the production of agglutinins, precipitins, and complement-fixing antibodies when inoculated into rabbits. It is proposed to deal with these antigenic responses in detail in a subsequent paper. For the present, it may be pointed out that the L and S sera referred to in this and the previous paper (Craigie and Wishart, 1936) were produced by inoculation with LS fraction. The S sera were prepared by the inoculation of LS fraction heated in order to inactivate the L component, while the L sera were derived from sera prepared by inoculation of untreated LS fraction by absorbing the S antibody from them with heated LS fraction.

Thermolability and Thermostability.—The demonstration of thermolabile and thermostable precipitable substances in vaccinia filtrates, or the LS fraction derived therefrom, is dependent on the use of sera which contain only one or other of two distinct precipitins. Both precipitins are present in the sera of rabbits during the 2nd week after vaccination but subsequently diminish or disappear. Hyperimmunization of the vaccinated animal at a later date with (a) elementary bodies or (b) LS fraction, provides, in most cases, sera of considerably higher titre than does vaccination alone. Both precipitins are developed in response to inoculation with either of these antigens. This may be shown by absorption of the serum with LS fraction heated at 70°C. for an hour, when the absorbed serum will be found to retain its capacity to precipitate unheated LS fraction although the precipitins for the heated fraction have been removed. Table IV provides an example of this phenomenon.

At this point the method of precipitin absorption used should be described. Prior to the absorption tests, Dean and Webb tests are set up to determine the

equivalent amounts of the serum and the antigen preparation. The serum and antigen are then mixed in the ratio indicated by this test, are held for 1 hour at room temperature, and then, after incubation for 1 hour at 50°C., the precipitate is removed by centrifugation. The controls required in tests on the absorbed serum include (a) a test for excess antigen by the addition of further precipitin and (b) a test for nonspecific inhibitory substances derived from the antigen preparation. In the latter test minimal amounts of antigen and serum which will give evident precipitation are added to a series of dilutions of the absorbed serum. This test is particularly necessary when crude filtrates are used for absorption, since otherwise inhibition of precipitation may be mistaken for evidence of absorption.

Sera, before being used as pure L or pure S sera, must be checked by means of absorption tests. Absorption is, of course, necessary in the case of L sera from which the S antibody must be removed. S sera, although prepared by injection of heated antigen, require to be checked by absorption in case L antibody, formed in response to earlier vaccination, has persisted in the serum. By means of pure L and pure S sera the difference in thermostability of the corresponding antigens may be readily demonstrated (Table II).

As Table II shows, the precipitable substance which reacts with one antibody is thermolabile at 56°C., while that which reacts with the other is stable at 99°C. These substances therefore correspond in their thermostability with the previously described L and S agglutinogens, and the L and S components of the LS antigen dissociated *in vitro* from elementary bodies. It has been the practice to carry out vaccinia precipitin reactions at 56°C. In view of the thermolability of the L substance the question arises as to whether 50°C. is not too high a temperature of incubation when precipitation of this substance is involved. Tests carried out at various temperatures have indicated that 50°C. promotes a greater extent of precipitation than 56°C. or 37°C. Table III shows the results obtained when a constant amount of antigen was mixed with various dilutions of serum and incubated at various temperatures.

As this table shows, L precipitation is weakened at 56°C., compared with that at 50°C., and is also decreased at temperatures lower than 50°C. The comparatively feeble precipitation which is obtained at cold room temperature is worthy of note since maximum complement fixation occurs at this temperature, presumably on account of the

greater antigen-antibody surface retained with delayed aggregation (Craigie and Wishart, 1934 b). Other tests carried out at 50°C. in which serum and antigen were preheated to 50°C. and also mixed and allowed to stand at room temperature for periods up to 1 hour showed no difference in the degree of precipitation. Apparently, therefore,

TABLE II
Thermostability of L and S Precipitinogens

Treatment of LS fraction 238	L serum 105, 1 in 30, and LS fraction diluted					S serum 335, 1 in 40, and LS fraction diluted				
	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600
Untreated	+++	+++	++	+	—	+++	+++	++	+	—
1 hr. at 56°C.	—	—	—	—	—	+++	+++	++	+	—
1 hr. at 70°C.	—	—	—	—	—	+++	+++	++	+	—
15 min. at 99°C.	—	—	—	—	—	+++	+++	++	+	—
	—	—	—	—	—	+++	+++	++	±	—

Plus signs indicate amount of precipitation.

— = no precipitation or opacity.

Tests read after incubation at 50°C. for 1 hour.

TABLE III
Influence of Temperature of Incubation on Precipitation

Temperature of incubation for 18 hrs.	LS fraction 234, 1 in 300, and LS serum 329 diluted				LS fraction 234, 1 in 300, and L serum E6 diluted				Heated LS fraction 225, and S serum 243 diluted		
°C.	1 in 20	1 in 40	1 in 80	1 in 160	1 in 20	1 in 40	1 in 80	1 in 160	1 in 20	1 in 40	1 in 80
56	++++	++++	++	+	+++	±	—	—	+++	+	—
50	++++	++++	+++	++	+++	++	±	—	+++	±	—
37	++++	+++	+++	++	+++	+	—	—	+++	±	—
18	+++	++	++	+	+++	±	—	—	++	—	—
5	+	+	—	—	±	—	—	—	±	—	—

50°C. is a suitable temperature for the incubation both of L and S precipitin tests and the preliminary period of contact at room temperature, up to 1 hour, has no significant effect. For complete precipitation with small amounts of antigen or antibody a period of at least 8 hours is necessary and it has been found convenient to incubate the tests overnight in open tubes in a closed water bath at 50°C.

Relationship of L and S Antigens.—By heating the LS fraction, the L substance may be eliminated, thus yielding preparations which apparently contain only the S substance. In the hope that corresponding preparations containing only the L substance might be obtained, attempts were made to absorb the S substance from the LS fraction by means of pure S serum. It was found, however, that as in the case of the dissociated LS antigen (Craigie and Wishart, 1936) both substances were removed from solution when either pure S or pure L sera were added. As suggested in the previous paper, this probably indicates that the L and S antigens are different antigenic

TABLE IV

Treatment of serum 366*	Serum titre for					
	Elementary bodies		Dissociated antigen		LS fraction	
	N	H	N	H	N	H
Unabsorbed	1 in 640	1 in 320	1 in 80	1 in 40	1 in 40	1 in 40
Absorbed with heated elementary bodies	1 in 640	0	1 in 40	0	n.t.	n.t.
Absorbed with LS fraction	0	0	0	0	0	0
Absorbed with heated LS fraction	1 in 640	0	1 in 80	0	1 in 40	0

N = untreated.

H = heated for 1 hour at 70°C.

0 = negative 1 in 5.

n.t. = not tested.

* Obtained by hyperimmunization with LS fraction.

components of a complex LS antigen rather than two independent antigens. The corresponding antibodies, however, are independent and sera containing either may be readily obtained. The relative amounts of L and S antibody formed in response to inoculation with LS antigen vary in different rabbits. In contrast to this, the titres of L and S substances in filtrates, or dissociated from elementary bodies, show an almost constant ratio to each other irrespective of the actual amounts present.

Scrological Relationships to the Elementary Body Agglutinogens.—Evidence that the L and S antibodies which precipitate the L and S

soluble substances are also involved in the agglutination of washed elementary bodies has been obtained by absorption tests. A number of such tests have yielded results similar to those shown in Table IV which indicate that the LS antigen found in solution in fresh vaccine pulp is serologically similar to antigens retained by washed elementary bodies.

As illustrated in Table IV, complete removal of agglutinins results on the addition of the equivalent amount of LS fraction to many sera prepared by hyperimmunizing rabbits with LS fraction. In contrast with this, it has been found that LS fraction may not remove all agglutinins from sera prepared by hyperimmunization with infective elementary bodies (Table V).

TABLE V

Serum No.	Treatment of serum	Serum titre for		
		Elementary bodies	LS fraction	Heated LS fraction
204*	Unabsorbed	1 in 1280	1 in 80	1 in 40
	Absorbed with LS fraction	1 in 320	0	0
331*	Unabsorbed	1 in 1280	1 in 160	1 in 80
	Absorbed with LS fraction	1 in 320	1 in 5	1 in 5

0 = negative 1 in 5.

*Obtained by hyperimmunization with infective elementary bodies.

These residual agglutinins presumably indicate additional antigens in the elementary bodies and it is suggestive that sera prepared with LS fraction are usually deficient in respect of these agglutinins. It has also been found that different elementary body suspensions vary considerably in their sensitivity to agglutination by these residual agglutinins. Further investigation of these additional antigens is being undertaken.

DISCUSSION

The observations reported in this and in two previous papers (Craigie and Wishart, 1934 a, 1936) indicate that the agglutination and precipitin reactions of vaccinia are for the most part referable to

the LS antigen. This antigen produces two distinct kinds of antibody, one of which reacts with its thermolabile L component, the other with its thermostable S component. Each of these antibodies may produce agglutination or precipitation according to whether the antigen is present on elementary bodies or is in a state of solution. Agglutinin and precipitin absorption tests provide evidence that the LS antigen occurs (a) on the elementary bodies of vaccinia, (b) in solution in fresh suspensions of dermal vaccine. *In vitro*, the LS antigen may be observed to dissociate from the elementary bodies and it would seem reasonable to assume that the LS antigen found in solution in fresh dermal vaccine represents antigen dissociated from the virus. However, it is not known whether the LS antigen of dermal vaccine exists *in vivo* in the state of solution in which it is recovered or whether its liberation from the virus is brought about by manipulation of the vaccine pulp. The question of the virus origin of this antigen has been previously discussed (Craigie and Wishart, 1936) when it was pointed out that the highly resistant elementary bodies which may be isolated from vaccinia-infected tissue do not, from a functional point of view, represent the only form in which vaccine virus may exist. It was suggested that the LS antigen was a specific product of vaccine virus, not in the resting, resistant state shown by elementary bodies *in vitro*, but in the active phase associated with its proliferation in a suitable cellular environment.

The LS antigen is specifically related to the viruses of vaccinia and variola. It has been found in dermal vaccine extracts of the guinea pig and calf as well as the rabbit (Craigie and Wishart, 1935). In the rabbit the C.L. strain of vaccine virus has been principally used in these investigations but unpublished observations show that the same LS antigen is formed by other strains, such as the Armstrong testicular strain, the Mulford Z encephalitogenic strain, and variola strains adapted to the rabbit. In all circumstances in which this antigen is found, L and S components are present in an approximately constant ratio unless the material has been exposed to conditions which inactivate the L component.

The thermolability of the L antigen at 56°C. is of interest. In this respect it resembles the Vi agglutinin of *B. typhosus* but it differs in regard to its stability to ether. The L antigen of vaccinia is stable

to ether but the Vi agglutinin of *B. typhosus* is inactivated by this reagent. Craigie and Tulloch (1931) found that the flocculable substance separated by CO₂ precipitation from vaccinia extracts was heat-labile at 56°C. It is now known that both L and S components are precipitated by CO₂ and the previous failure to detect the latter is to be attributed to the use of sera in concentrations in which only L precipitin was present in effective concentrations. When the LS antigen is heated at 70°C. the L component is destroyed but the S component remains antigenic. A few tests indicate that the antigenicity of the S component is considerably reduced by steaming it and the heat-stable precipitable substances described by Wilson Smith (1932) and by Ch'en (1934) were obtained by boiling vaccinia extracts in acid and these were found by them to be nonantigenic. It seems probable that the carbohydrate obtained by Ch'en represents a polysaccharide haptene of the S component of the LS antigen, but further enquiry in this direction is desirable. Hughes, Parker, and Rivers (1935) applied the Molisch test and precipitin reaction to each of seven wash waters obtained during the purification of a batch of elementary bodies, since previous analyses of washed elementary bodies showed comparatively small amounts of carbohydrate. Examination of the wash waters from the elementary bodies showed that the Molisch and precipitin tests roughly paralleled each other and that the amounts of carbohydrate and precipitable substance progressively decreased in successive wash waters. Further study of the carbohydrate fraction in relation to the LS antigen dissociated from elementary bodies should be of interest.

The LS antigen is probably not the only antigen of vaccinia virus which reacts *in vitro*. In addition to a neutralizing antibody which inactivates the elementary bodies *in vitro*, agglutinins distinct from the L and S agglutinins are found in some antivaccinia sera. While the terms L and S were first used in reference to the agglutinogens of elementary bodies it is no longer desirable to define them in this way, at least until further investigation of the additional agglutinogens has been carried out. LS fraction prepared and preserved by the method described in this paper appears to be deficient in these additional antigens and the L and S antigens are preferably to be defined as the thermolabile and thermostable components of the LS antigen obtained in this way.

We are indebted to Mr. M. D. Orr, Connaught Laboratories, for his kindness in undertaking the nitrogen estimations recorded in Table I.

CONCLUSIONS

1. Thermolabile (L) and thermostable (S) antigens occur in solution in suspensions of vaccine pulp.
2. These antigens are also present on the elementary bodies of vaccinia and participate in the agglutination of these bodies by anti-vaccinia serum.

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STUDIES ON THE SOLUBLE PRECIPITABLE SUBSTANCES OF VACCINIA

III. THE PRECIPITIN RESPONSES OF RABBITS TO THE LS ANTIGEN OF VACCINIA

By F. O. WISHART, M.D., AND JAMES CRAIGIE, M.B., CH.B.
(From the Connaught Laboratories and School of Hygiene, University of Toronto,
Toronto, Canada)

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In previous papers, Craigie and Wishart (1934 a; 1936 a, b) reported that the soluble precipitable substances of vaccinia and the corresponding agglutinogens of the elementary bodies have a complex antigenic composition. Two antigenic components have been differentiated and these show a marked difference in their thermostability.

These have been termed the L (labile) and S (stable) components, respectively. The former is labile at 56°C. and the latter is stable at temperatures up to 100°C. Absorption tests have shown that each component reacts with its homologous antibody, whether present in solution or fixed to the elementary body, and that the L and S antibodies are distinct and may be obtained separately. The L and S antigens, on the other hand, behave *in vitro* as if components of a complex LS antigen.

The *in vitro* analysis of the antigens of vaccinia has been accompanied by extensive observations on the precipitin and agglutinin responses of rabbits to the injection of elementary bodies and the soluble antigens of vaccinia. These observations will be summarized in this paper. Since the agglutinin or precipitin response of over a hundred rabbits to hyperimmunization has been followed it is possible to give only representative examples, indicating at the same time the extent of variations in response which have been encountered. This paper also supplements the two previous papers in this series in providing further information regarding the L and S precipitin sera used in the investigation of the soluble precipitable substances of vaccinia.

EXPERIMENTAL

Methods

The vaccine virus preparations used have been chiefly obtained with the C.L. strain of lapine (Craigie and Wishart, 1934 a). All data given in tabular form in this paper relate to this strain. Where details of methods are omitted they will be found in papers to which reference is given. All sera, following their separation from the blood clot, were heated for 45 minutes at 56°C. as a precaution against the antagonistic effect on precipitation demonstrable with low titre precipitin sera (Craigie, 1932). Agglutinins were titrated by the method described by Craigie and Wishart (1934 a), and the complement-fixing antibodies by the method described by the same authors (1934 b). Suitable dilutions of Seitz filtrates of lapine or LS fraction (Craigie and Wishart, 1936 b) were used for the titration of precipitins, the tests being incubated for 18 hours at 50°C.

Filtrate or LS fraction heated for 1 hour at 70°C. was used for the direct titration of S antibody, while unheated antigen was used to obtain the titre of the predominant antibody. Both the LS and S antigens are precipitated by S antibody, and an LS titre which does not definitely exceed the S titre offers no clue to the L antibody titre. Before the L titre can be determined the S antibody must be removed from the serum by the application of an equivalent amount of S antigen (Craigie and Wishart, 1936 b), and this was resorted to when necessary. On the other hand, it has been found that an LS titre more than twice as great as the S titre may be provisionally accepted as the L titre provided that the test LS and S antigens contain similar amounts of S antigen.

Antibody Formation Following Vaccination

Table I and the first portion of Fig. 1 show the development of agglutinins, precipitins, and complement-fixing antibodies following dermal vaccination. Only unheated elementary bodies and lapine filtrate were used in the serum titrations, and the figure and Table I therefore indicate only the titre of the predominant type of antibody.

The antibody curves shown are typical in respect of the times of first appearance and maximum presence of antibody. The agglutinin, complement-fixing, and precipitin titres, shown in Fig. 1, however, are higher than is usually the case. Following vaccination the maximum agglutinin and complement-fixing titres generally range from 1 in 160 to 1 in 640 while precipitin titres range from 1 in 2.5 to 1 in 20. The precipitins (Fig. 1) show a relative increase in their ratio to the agglutinins from the 7th to the 16th day. This is probably more apparent than real, reflecting the inaccuracies of precipitin tests car-

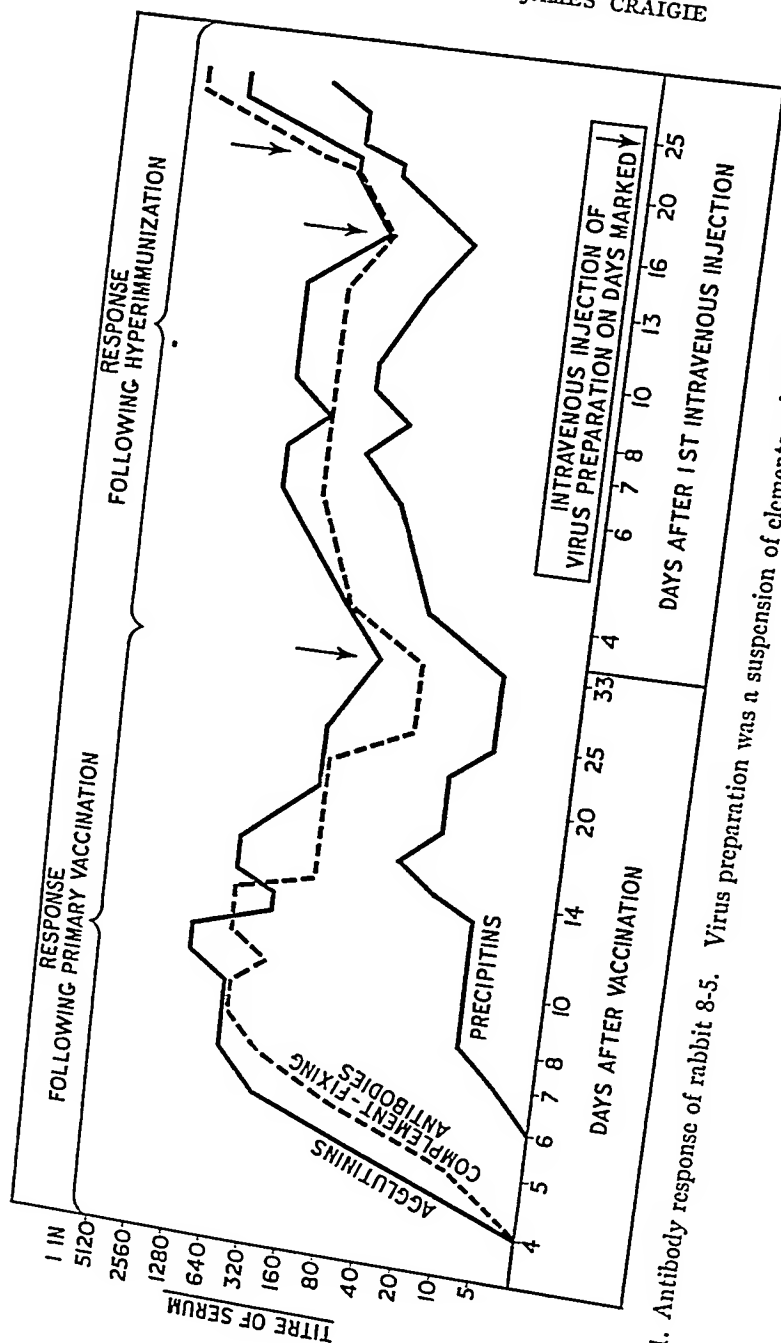


FIG. 1. Antibody response of rabbit 8-5. Virus preparation was a suspension of elementary bodies in lapine Seitz filtrate.

ried out with crude lapine filtrates. Other series of vaccinated rabbits have shown a close parallelism between the precipitins, agglutinins, and complement-fixing antibody, and further observations are required on the antibody response to vaccination, embracing estimations of L and S antibodies with the methods now available. It is known, however, that both L and S antibodies develop after vaccination. As shown in Fig. 1, the antibodies exhibit a progressive decrease in the 3rd week following vaccination and this continues. Rabbits show a

TABLE I
Response to Dermal Vaccination

Rabbit No.	Day after vaccination	Agglutinin titre	Complement-fixing titre	Precipitin titres
7-5	4th	1 in 5	<1 in 20	<1 in 2.5
	7th	1 in 320	1 in 160	1 in 2.5
	10th	1 in 320	1 in 160	1 in 10
	16th	1 in 160	1 in 80	1 in 20
	33rd	1 in 20	1 in 20	1 in 10
9-7	4th	<1 in 5	<1 in 20	<1 in 2.5
	7th	1 in 40	1 in 20	<1 in 2.5
	10th	1 in 320	1 in 320	<1 in 2.5
	16th	1 in 160	1 in 80	1 in 2.5
	33rd	1 in 80	1 in 40	<1 in 2.5
9-9	4th	1 in 40	<1 in 20	<1 in 2.5
	7th	1 in 640	1 in 320	<1 in 2.5
	10th	1 in 320	1 in 160	1 in 5
	16th	1 in 160	1 in 80	1 in 5
	33rd	1 in 40	1 in 20	1 in 5

considerable variation in the amounts of antibodies which persist 1 or 2 months following vaccination. Precipitins generally disappear in 4 to 6 weeks while small amounts of agglutinins may persist for a longer period.

Infective virus is not essential for the formation of antibodies in the normal rabbit, since intravenous injection of both elementary bodies rendered noninfective by heat or formalin and virus-free Seitz filtrates produce agglutinins, complement-fixing antibodies, and precipitins (Craigie, 1934). Certain differences, however, were found in the

responses due to vaccination and to inoculation with noninfective preparations. Reinvestigation with special reference to L and S antibodies will be necessary before the significance of these differences can be assessed.

Antibody Formation on Hyperimmunization

The latter part of Fig. 1 shows that a further development of antibodies occurs when immune rabbits are inoculated with virus preparations and that higher antibody titres may be obtained by hyperimmunization than by vaccination alone. In this instance the agglutinogens and precipitinogens of vaccinia were both injected intravenously, the injections consisting of elementary bodies suspended in a Seitz filtrate of lapine which contained the soluble precipitable substances. This fractionation and subsequent pooling of the antigens was necessary in order to obtain the total antigens of vaccinia in a form suitable for intravenous injection. Apart from variation in degree of antibody response to vaccination, rabbits show a considerable variation as regards the later rate of fall of antibody titre. Reference to the tables in this paper will show that the sera of rabbits, 2 to 3 months after vaccination, contain no demonstrable amounts of precipitin. At this interval after vaccination the agglutinins either show a very low titre or are not demonstrable. Hyperimmunization appears to be equally effective whether 3 weeks or 3 months have elapsed since vaccination, and has been extensively employed in the study of the antigenic qualities of the LS antigen. While it might be objected that this method indicates only the effectiveness of the preparations as secondary stimuli, the use of normal rabbits is open to more serious objection unless very rigid isolation of the animals and extensive testing of the preparations for the complete absence of infective virus is practised. Duran-Reynals (1931) has pointed out that the risk of infection of rabbits with vaccine virus is increased by intravenous injection and the taking of blood samples. We have, on a number of occasions, observed the development of agglutinins for elementary bodies in normal rabbits merely placed in the same room with vaccinated rabbits, but not in proximity to them. This formation of agglutinins is accompanied by the development of immunity, probably as a result

of subclinical infection *via* the respiratory tract. Normal rabbits can, therefore, only be employed in the investigation of antigenic qualities of vaccinia antigens if fallacies due to infection with the virus are excluded by rigid isolation, adequate testing of the inocula for absence of infective virus, and the provision of an adequate number of uninoculated control animals. For this reason we have resorted to hyperimmunization of immune rabbits, permitting a sufficient interval for the disappearance of precipitins to elapse after vaccination.

Hyperimmunization with Heated Elementary Bodies

As previously reported (Craigie and Wishart, 1934 a) vaccination or hyperimmunization with infective elementary bodies leads to the production of L and S agglutinins and, as later found, L and S precipitins also. Heated elementary bodies were found to absorb only the S antibody. Table II provides examples of the increase of precipitin which results when rabbits are hyperimmunized with elementary bodies heated at 90°C. The responses shown in this table are representative of a larger series.

Samples of serum indicated in Table II were examined by absorption and no L antibody was found. From this and other observations we conclude that exposure to 70°C. for an hour completely inactivates the L antigen while the S antigen remains antigenic after being heated at 90°C. for 1 hour. Although the L antigen is destroyed at 56°C. as far as its reactivity *in vitro* is concerned, it seems preferable to use a temperature of 70°C. to ensure destruction of the last traces of this antigen when the material is to be used for serum production.

Hyperimmunization with Infective Elementary Bodies and Dissociated Antigen

Table III shows the responses of six rabbits to hyperimmunization with infective elementary bodies.

The first injection consisted of antigen dissociated from a washed suspension of elementary bodies and in this experiment only a slight response occurred in three of the rabbits. In other experiments where further injections of dissociated antigen were given, a more definite formation of L and S antibodies has resulted. Compared with LS fraction, dissociated antigen preparations contain considerably less

TABLE II

Responses to Injection of Heated Elementary Bodies

Rabbit No. and precipitin titre

Date of injection	Injection (intravenously)	Date of serum test	D3-13				D3-16				D3-22				D3-28			
			LS		S		LS		S		LS		S		LS		S	
1935		1935																
Mar. 16	Dermal vaccination	Apr. 5	1 in 5	1 in 2.5	0	1 in 5	1 in 80	1 in 160	1 in 20	1 in 5	0	1 in 80	1 in 80	1 in 2.5				
Apr. 5	0.5 cc. E.B. 90°C.	Apr. 10	1 in 80	1 in 80	1 in 80	1 in 80	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160	1 in 160				
Apr. 10	0.5 cc. E.B. 90°C.	Apr. 15	1 in 80	1 in 160	1 in 40	1 in 40	1 in 40	1 in 40	1 in 20	1 in 20	1 in 20	1 in 20	1 in 20	1 in 20				
Apr. 15	0.5 cc. E.B. 90°C.	Apr. 23	1 in 40	1 in 40	1 in 40	1 in 40	1 in 40	1 in 40	1 in 20	1 in 20	1 in 20	1 in 20	1 in 20	1 in 20				
May 10	0.75 cc. E.B. 70°C.	May 16	1 in 40	1 in 80	—	1 in 80	1 in 40	1 in 40	1 in 20	1 in 20	1 in 20	1 in 20	1 in 20	1 in 20				
May 16	1.0 cc. E.B. 70°C.	May 28	1 in 80	1 in 160	—	—	—	—	—	—	—	—	—	—				

90°C. = suspension heated at 90°C. for 1 hour.

E.B. = elementary body suspension. 0 = titre less than 1 in 2.5.

TABLE III

Response to Injection of Infective Elementary Bodies

Rabbit No. and precipitin titre

		Rabbit No. and precipitin test		Test antigen =											
Date of injection	Infection (intravenously)	Date of serum test	D2-26		D2-31		D2-32		D2-33		D2-38		D2-41		
			LS	S	LS	S	LS	S	LS	S	LS	S			
1935		1935													
Jan. 15	Dermal vaccination	Feb. 8	0	0	1 in 40	1 in 10	1 in 10	0	1 in 5	0	1 in 20	0	0	0	
Feb. 8	D.A. 1 cc.	Feb. 15	0	0	1 in 80	1 in 40	1 in 10	1 in 10	1 in 5	1 in 20	1 in 20	0	1 in 20	0	
Feb. 15	E.B. 1 cc.	—	—	—	—	—	—	—	—	—	—	—	—	—	
Feb. 22	E.B. 1 cc.	Feb. 27	1 in 80	1 in 20	1 in 80	1 in 40	1 in 40	1 in 40	1 in 20	1 in 160	1 in 80	1 in 20	1 in 160	1 in 40	
Feb. 27	E.B. 1 cc.	Mar. 5	1 in 160	1 in 40	1 in 320	1 in 80	1 in 80	1 in 80	1 in 20	1 in 320	1 in 80	1 in 160	1 in 320	1 in 160	

Test antigen =

D.A. = dissociated antigen from washed elementary bodies. E.B. = washed elementary body suspension.

0 = titre less than 1 in 2.5.

S. Seitz filtrate heated at 70°C.

precipitable substance (Craigie and Wishart, 1936 a) and we consider that the feeble response to injection of dissociated antigen is referable to its small content of antigen. The subsequent injections in the experiment shown in Table III consisted of infective elementary bodies and these brought about a considerable rise in precipitin titre. It will be noted that the LS titres (*i.e.* for untreated filtrate), in contrast to those produced by heated elementary bodies (Table II) were significantly higher than the S titre (*i.e.* for heated filtrate). Comparison of Table III with Table IV will show that rather higher precipitin titres were produced with infective elementary bodies than with LS antigen. This slightly greater effectiveness of elementary bodies in evoking a response seems to be generally true.

Hyperimmunization with LS Fraction

Seitz filtrates of lapine have been used for the production of precipitin sera and, more recently, the LS fraction (partially purified LS antigen) which is derived from Seitz filtrates (Craigie and Wishart, 1936 b). Tables IV and V show the responses of rabbits to intravenous hyperimmunization with LS fraction and S (*i.e.* heated LS) fraction respectively.

It will be noted that the doses of antigen were graded and it is believed that this procedure, with a 5 to 6 day spacing of the doses, generally evokes the maximum response of which the individual rabbit is capable. The doses are expressed in arbitrary units, one unit representing that amount of LS antigen which will just give perceptible precipitation with the optimum amount of precipitin in a volume of 0.5 cc. The variations in degree of response shown in Tables IV and V may be taken as representative. Occasionally a maximum titre of 1 in 320 has been encountered and on the other hand a few rabbits have failed to attain either an L or S titre greater than 1 in 10. A comparison of Tables IV and V will show that only after injection with unheated LS fraction may the LS titre exceed the S titre. By means of absorption tests it was shown that the rabbits inoculated with unheated LS fraction developed both L and S antibodies, while those inoculated with S fraction developed S antibodies only. The L antigen, therefore, is completely inactivated on heating LS fraction for 1 hour at 70°C.

As shown in Tables IV and V variations occur in the time taken

TABLE IV

Response to Injection of LS Fraction

Date of Injection	Injection LS fraction (intravenously)	Date of serum test	Rabbit No. and precipitin titre					
			E9		E1-0		E1-1	
			LS	S	LS	S	LS	S
1935		1935						
July 10 to 11	Dermal vaccination	Oct. 12	0	0	0	0	0	0
Oct. 12	5 units	Oct. 18	0	0	1 in 5	1 in 10	1 in 5	1 in 5
Oct. 18	20 units	Oct. 23	1 in 80	1 in 20	1 in 20	1 in 40	1 in 20	1 in 10
Oct. 23	20 units	Oct. 28	1 in 80	1 in 40	1 in 40	1 in 40	1 in 40	1 in 10
Oct. 29	100 units	Nov. 5	1 in 80	1 in 40	1 in 20	1 in 160	1 in 80	1 in 10
Oct. 29	100 units	Nov. 13	—	—	1 in 40	—	1 in 80	1 in 10
Nov. 6	100 units	Nov. 19	—	—	1 in 10	—	1 in 10	1 in 5
Nov. 14	400 units							

0 = titre less than 1 in 2.5.

TABLE V

Response to Injection of S Fraction

Date of Injection	Injection S fraction (intravenously)	Date of serum test	Rabbit No. and precipitin titre					
			E5		E1-4		E1-5	
			LS	S	LS	S	LS	S
1935		1935						
July 9	Dermal vaccination	Oct. 12	0	0	0	0	0	0
Oct. 12	5 units	Oct. 18	1 in 5	1 in 10	0	1 in 5	1 in 5	1 in 10
Oct. 18	20 units	Oct. 23	1 in 40	1 in 80	1 in 40	1 in 20	1 in 40	1 in 80
Oct. 23	20 units	Oct. 28	1 in 80	1 in 160	1 in 40	1 in 10	1 in 40	1 in 80
Oct. 29	100 units	Nov. 5	1 in 80	1 in 80	1 in 40	1 in 20	1 in 80	1 in 40
Nov. 6	100 units	Nov. 13	1 in 80	1 in 80	1 in 40	1 in 20	1 in 40	1 in 20
Nov. 14	400 units	Nov. 19	1 in 40	1 in 40	1 in 20	1 in 10	1 in 20	1 in 20

Test antigens—LS = LS fraction, S = LS fraction heated at 70°C.

S fraction = LS fraction heated at 70°C. for 1 hour.

0 = titre less than 1 in 2.5.

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TABLE IV
Response to Injection of LS Fraction

Date of injection	Injection LS fraction (intravenously)	Date of serum test	Rabbit No. and precipitin titre							
			E9		E1-0		E1-1		E1-2	
			LS	S	LS	S	LS	S	LS	S
1935		1935								
July 10 to 11	Dermal vaccination	Oct. 12	0	0	0	0	0	0	0	0
Oct. 12	5 units	Oct. 18	0	0	1 in 5	1 in 5	1 in 10	1 in 5	0	1 in 5
Oct. 18	20 units	Oct. 23	1 in 80	1 in 20	1 in 20	1 in 20	1 in 40	1 in 20	1 in 20	1 in 5
Oct. 23	20 units	Oct. 28	1 in 80	1 in 40	1 in 40	1 in 40	1 in 40	1 in 20	1 in 40	1 in 10
Oct. 29	100 units	Nov. 5	1 in 80	1 in 40	1 in 40	1 in 20	1 in 160	1 in 80	1 in 40	1 in 10
Nov. 6	100 units	Nov. 13	—	—	1 in 40	1 in 20	—	—	1 in 80	1 in 10
Nov. 14	400 units	Nov. 19	—	—	1 in 10	1 in 10	—	—	1 in 10	1 in 5

Test antigens—LS = LS fraction, S = LS fraction heated at 70°C. for 1 hour. 0 = titre less than 1 in 2.5.

TABLE V
Response to Injection of S Fraction

Date of injection	Injection S fraction (intravenously)	Date of serum test	Rabbit No. and precipitin titre							
			E8		E1-4		E1-5		E1-8	
			LS	S	LS	S	LS	S	LS	S
1935		1935								
July 9	Dermal vaccination	Oct. 12	0	0	0	0	0	0	0	0
Oct. 12	5 units	Oct. 18	1 in 5	1 in 10	0	1 in 10	0	1 in 5	1 in 5	1 in 10
Oct. 18	20 units	Oct. 23	1 in 40	1 in 80	1 in 20	1 in 40	1 in 10	1 in 40	1 in 40	1 in 80
Oct. 23	20 units	Oct. 28	1 in 80	1 in 160	1 in 40	1 in 40	1 in 10	1 in 40	1 in 40	1 in 80
Oct. 29	100 units	Nov. 5	1 in 80	1 in 80	1 in 40	1 in 40	1 in 20	1 in 80	1 in 80	1 in 80
Nov. 6	100 units	Nov. 13	1 in 80	1 in 80	1 in 40	1 in 40	1 in 20	1 in 20	1 in 40	1 in 40
Nov. 14	400 units	Nov. 19	1 in 40	1 in 40	1 in 20	1 in 20	1 in 10	1 in 20	1 in 20	1 in 20

S fraction = LS fraction heated at 70°C. for 1 hour. Test antigens—LS = LS fraction, S = LS fraction heated at 70°C. for 1 hour. 0 = titre less than 1 in 2.5.

for the maximum titres to be reached. These tables also illustrate a general finding, that after several injections of antigen the ability of the rabbit to respond is impaired and that thereafter the antibody titre falls in spite of an increase in the dose of antigen. A good deal of variation occurs in the ratio of L to S antibody produced by inoculation of LS antigen, and S antibody may predominate. L sera which are necessary for identification of the L antigen are prepared by absorbing out the S antibody present in a serum containing both antibodies. In an endeavour to obtain high titre L sera and facilitate the absorption of S antibody from them, attempts were made to increase the relative proportion of L antibody by adding an excess of S serum to LS fraction prior to injecting it. While this procedure does not necessarily completely prevent formation of S antibody the results obtained certainly suggest that it is of value in generally decreasing the S response without affecting the development of L antibody.

In general, elementary body suspensions are rather more effective than LS fraction in promoting L precipitin formation. However, in view of evidence that infective elementary bodies possess other agglutinogens as well as the LS antigen (Craigie and Wishart, 1936 b), the serological behaviour of sera produced by their inoculation should be interpreted with caution. Until further information regarding these additional antigens has been obtained, the antibodies found in sera produced by the inoculation of LS fraction are to be preferred for the identification of the L and S antigens.

SUMMARY AND CONCLUSIONS

Observations on antibody production in the rabbit in response to the injection of the LS antigen of vaccinia have shown that this antigen retains its ability to stimulate the production of L and S antibodies, not only when it is in a state of solution as found in lapine Seitz filtrates, but also after it has been partially purified. As reported in previous papers, both the L and S antibodies participate (a) in the true agglutination of washed elementary bodies, (b) in the precipitation of the soluble LS antigen found in fresh vaccine suspensions or dissociated *in vitro* from washed elementary bodies. The difference in the thermostability of the L and S components of the LS antigen as far as their reactivity *in vitro* is concerned, holds in respect of their capac-

ity to stimulate antibody production. Heating at 70°C. invariably abolishes the antigenicity of the L component while the S component remains antigenic after being heated to 90°C.

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IMMUNOLOGICAL STUDIES ON A NEW PREPARATION OF TYPE SPECIFIC POLYSACCHARIDE FROM PNEUMOCOCCUS TYPE I

By BACON F. CHOW, Ph.D.

(From the Department of Biochemistry, Peiping Union Medical College, Peiping, China)

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The isolation of polysaccharide from the three most common types of pneumococci by Avery and Heidelberger (1-3) has made it possible to explain the biological type specificity on a chemical basis and is a definite advance in the study of the antigenic structure of the bacteria. Since the isolation both European (4) and American (5-7) laboratories have also obtained polysaccharides from Type I Pneumococcus, which are apparently different from Avery and Heidelberger's soluble specific substance. Thus several names have been given by various authors to the polysaccharides of Type I Pneumococcus. Fortunately, the general confusion has been greatly clarified by the recent paper of Avery and Goebel (8). They isolated from the same bacteria a polysaccharide in a more complete form, through the omission of an alkali treatment in their preparation. This acetyl polysaccharide, as they called it, has labile acetyl groups attached to the soluble specific substance. The antigenicity in mice depends on the presence of the acetyl group.

In this report, we shall show that an even more complete polysaccharide can be isolated from the autolyzed broth culture of Type I Pneumococcus by slightly modifying Avery and Goebel's method.

1. Preparation of the Polysaccharide of Pneumococcus Type I from the Autolyzed Broth Culture

Culture.—The Pneumococcus Type I strain used for this work was obtained from The Rockefeller Institute, New York. Its virulence was such that 10^{-8} cc. of an 18 hour broth culture killed mice within 48 hours.

Composition of Culture Medium.—Virulent Type I pneumococci were grown in meat infusion broth of pH 7.6 containing 0.5 per cent NaCl and 1.0 per cent dextrose.

30 liters of the broth culture was seeded with Pneumococcus Type I. After

12 hours of incubation at 37°C., 10 cc. of a 10 per cent sterile glucose solution was added to each liter of broth in order to increase the growth of bacteria. After 7 or 8 days, most of the bacteria had autolyzed. The pH of the autolyzed broth was 5.2. The culture was centrifuged to get rid of the bacterial debris and the unautolyzed cells. The slightly opalescent supernatant was concentrated to 3 liters *in vacuo*, the temperature of the culture being maintained below 37°C. 4 liters of alcohol (95 per cent) was added to the concentrated broth culture. After standing for 30 hours, the supernatant liquid was siphoned off and the precipitate was packed by centrifuging in four 250 cc. bottles. The precipitate in each bottle was washed with 50 cc. of water and recentrifuged. The washing was continued until the supernatant gave only a faint Molisch test. The combined supernatants were concentrated *in vacuo* to 600 cc. and cooled with ice. 70 cc. of 50 per cent trichloroacetic acid was added, and the suspension was centrifuged. The precipitate was washed 2 or 3 times with cold 10 per cent trichloroacetic acid. The combined supernatants and washings were again concentrated *in vacuo* to 200 cc. 10 volumes of acetone were added to precipitate the polysaccharide. After standing overnight the clear supernatant was siphoned off and the precipitate was collected by centrifugation. The polysaccharide was repeatedly washed with 20 cc. portions of water and a small amount of insoluble protein residue was discarded. The combined washings were again reprecipitated with 10 volumes of acetone. The polysaccharide was again washed with water and precipitated with acetone. This process was repeated 4 or 5 times. Finally, the clear polysaccharide solution was rapidly dialyzed in the cold against 0.001 N HCl. When the dialysis was complete, the polysaccharide was precipitated with 10 volumes of acetone, centrifuged, and dried with acetone and ether. The yield was 1.1 gm. The product was white. In 1 per cent solution it gave strong positive Molisch, but negative biuret, Millon's, or xanthoproteic tests.

2. Immunological Properties of the Newly Prepared Polysaccharide

It was found that when Type I antipneumococcus rabbit serum was absorbed with the acetyl polysaccharide, the absorbed serum still effectively protected mice from an otherwise fatal dose of Type I pneumococci. This fact means that either there is more than one kind of protective antibody in the rabbit antiserum or that the acetyl polysaccharide is still not in the natural and most complete form, or possibly both. The following experiments will show that our polysaccharide is still more complete than the acetyl polysaccharide, for it possesses the immunological properties of the latter, and it will also precipitate an antipneumococcus Type I rabbit serum absorbed with the acetyl polysaccharide.

Precipitin Reaction of the New Polysaccharide in Antipneumococcus Rabbit and Horse Sera, Absorbed and Unabsorbed by Acetyl Polysaccharide

It was shown elsewhere (9) that Type I antipneumococcus rabbit serum absorbed with the acetyl polysaccharide would still react with our polysaccharide and that our polysaccharide precipitated about 3 times as much of antibody from the immune rabbit serum as the acetyl polysaccharide.

It was therefore of interest to ascertain whether Type I antipneumococcus immune horse serum previously absorbed with the acetyl polysaccharide would react with the new polysaccharide. Our results

TABLE I

Determination of the Total Antibody Content of an Immune Rabbit Serum by the Quantitative Agglutination Method

Experiment No.	Volume of serum	Total volume of heat-killed vaccine	Total protein in the precipitate	Protein in total vaccine	Agglutinin protein per cc. of serum
	cc.	cc.	mg.	mg.	mg.
A	1.00	6.00	14.6	2.10	12.5
B	1.00	8.00	13.7	1.44	12.3

confirmed the findings of Avery and Goebel (8) in that unlike the immune rabbit serum, the corresponding horse immune serum previously absorbed with the acetyl polysaccharide would neither react with our polysaccharide nor agglutinate Type I pneumococci.

This difference in the homologous types of immune rabbit and horse sera is not unexpected, for it is now well known that immune sera from these two species differ both chemically and immunologically (10-12).

Per Cent of the Total Antibody Protein in Immune Rabbit Serum Precipitable by Our Polysaccharide

It was of interest to find what portion of the antibody in the immune serum was precipitated by the acetyl and by our polysaccharides. To find the total antibody content of the immune serum, we have followed the method of quantitative agglutination of Heidelberger and Kabat (13). The results are shown in Table I.

The procedure used is as follows: In Experiment A, 1 cc. of the immune serum was pipetted into each of two 15 cc. centrifuge tubes. 1 cc. of heat-killed vaccine prepared according to Heidelberger and Kabat's direction was added to each tube. The mixture was kept at 0°C. for 48 hours and then centrifuged at 0°C. The precipitate was washed with 2 cc. of cold saline and to the supernatant was added another 1 cc. of the vaccine. The process was again kept at 0°C. for 48 hours, and centrifuged as before. The process was repeated until there was no appreciable agglutination 48 hours after the addition of vaccine. The precipitates were combined and analyzed for nitrogen.

In Experiment B, 1 cc. of the immune serum was used, but the vaccine was diluted twofold. Otherwise the procedure was exactly the same. It will be noticed from Table I that it only took 8 cc. of the diluted vaccine to carry down the same amount of agglutinin as 6 cc. of the undiluted. The results in the table are the average of duplication determinations.

TABLE II
Protective Action of Antipneumococcus Rabbit Serum (Type I) before and after the Absorption with the Newly Prepared Polysaccharide

Serum dilution	Unabsorbed		Absorbed with the polysaccharide	
	S	S	S	S
1:400	S	S	S	S
1:200	S	S	S	S
1:80	S	S	S	S
1:20	—	—	S	S
1:10	—	—	S	S

S = survival for at least 5 days after the inoculation of culture.
Control mice receiving no serum died 48 hours after the injection of 10^{-8} , 10^{-7} , 10^{-6} cc. of 18 hour broth culture.

The last column in Table I shows that there are 12.4 mg. of antibody protein in each cc. of the immune serum. From the same serum our polysaccharide precipitated some 4.35 mg. protein or 35 per cent of the total antibody, whereas the acetyl polysaccharide precipitated 1.34 mg. protein or 11 per cent.

Although the newly prepared polysaccharide is more complete, yet it does not remove all the protective antibodies from the immune serum. Table II shows that immune serum previously absorbed by our polysaccharide still afforded effective protection in mice against 500,000 M.L.D. of virulent Type I Pneumococcus. All mice received an intraperitoneal injection of 0.5 cc. of various dilutions of sera together with 0.5 cc. of 1:200 dilution of 18 hour broth culture of pneumococcus Type I.

In Table III, it may be seen that neither the acetyl nor our polysaccharides, removed all the antibody from immune rabbit serum.

Though the absorbed serum could still agglutinate Type I pneumococci, the agglutination titre of the serum absorbed with our polysaccharide was greatly reduced.

Antigenicity of the Polysaccharide in Mice and Rats

An important guide of the immunological properties of the polysaccharide from Type I Pneumococcus is its antigenic response in mice. Many investigators have definitely shown that their polysaccharides derived from Type I Pneumococcus lost their antigenicity when boiled with alkali. Avery and Goebel have shown that the loss of antigenicity is due to the loss of acetyl groups of the polysaccharide.

TABLE III

Agglutination of Type I Pneumococcus in Immune Rabbit Serum before and after Absorption with the Polysaccharides

Serum	Final dilution of sera							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Unabsorbed	+++	++++	++++	++++	++++	+++	++	±
Absorbed with the acetyl polysaccharide	++++	++++	++±	++	+	—	—	—
Absorbed with the newly prepared polysaccharide	++	++	+	±	—	—	—	—

It was therefore of interest to see whether our polysaccharide, which is apparently more complete, would also lose its antigenicity in mice.

0.5 cc. of 1:million dilution of the polysaccharide was injected intraperitoneally into each of a group of six mice. Another group of six mice received identical amounts of the same polysaccharide previously heated at 100°C. in 0.05 N NaOH for 30 minutes. Three doses were given to each group at the intervals of 3 days. 3 days after the last injection, the mice were given intraperitoneally 0.5 cc. each of a virulent culture of Type I Pneumococcus. The results are given in Table IV.

It is of interest to note that our preparation is apparently more resistant to alkali than those from other laboratories. However, it must not be taken to mean that alkali will not destroy the antigenicity of our polysaccharide, for a prolonged boiling in 0.05 N will convert the polysaccharide to serologically nonreactive substances.

POLYSACCHARIDE FROM PNEUMOCOCCUS TYPE I

The antigenic action of our polysaccharide in rats was also observed. Two rats weighing about 300 gm. each received intravenous injections through the tail veins, 0.5 cc. of 1:1,000,000 solution of the polysaccharide. Another pair received

TABLE IV
Antigenic Action in Mice of the Newly Prepared Polysaccharide before and after Alkali Treatment

Dilution of pneumococcus culture (Type I)	Normal mice		Immunized mice injected with the newly prepared polysaccharide		Immunized mice injected with the alkali-treated polysaccharide	
cc.						
10 ⁻⁴	—	—				
10 ⁻⁵	D 30	D 48	S	S	S	S
10 ⁻⁶	D 30	D 49	S	S	S	S
10 ⁻⁷	D 30	D 49	S	S	S	S
10 ⁻⁸	D 30	D 30	—	—	—	—

S = survived. D followed by a number = death occurred at the number of hours. — = not done.

TABLE V
Protection of Mice against Fatal Infections with Type I Pneumococci by Rat Sera Immunized with the New Polysaccharide

Serum dilution	Source of serum		
	From rats receiving a total of 1 cc. of 1:1,000,000 solution of the polysaccharide	From rats receiving a total of 1 cc. of 1:10,000 solution of the polysaccharide	From normal rats receiving no polysaccharide
Undiluted	S S		
1:10	S S	S S	
1:100	S S	S S	D 30 D 44 D 30 D 36 D 30 D 36

Virulence Control

Dilution of culture	Result	
cc.		
10 ⁻⁶	D 30	D 44
10 ⁻⁷	D 44	D 48
10 ⁻⁸	D 44	D 44

S = survival. D followed by number = death occurred at the designated number of hours.

0.5 cc. of 1:10,000. The injections were repeated after 3 days. 3 days after the second injection the rats were bled to death and the sera from the two groups were collected. That these rat sera protected mice from a lethal dose of Type I Pneumococcus can be seen from Table V.

Antigenicity of the New Polysaccharide in Rabbits

None of the polysaccharides prepared in different laboratories has been reported to be antigenic in rabbits. It was therefore interesting to ascertain the antigenicity of our polysaccharide in rabbits. For that purpose, six rabbits were given intravenously three daily injections of 1 cc. of the polysaccharide (1:1000 solution), followed by a rest period of 3 days. Three courses of injection were given in all, so that each rabbit received a total amount of 9 mg. After the third course of injections, the rabbits were bled from the heart and the sera were tested for the presence of type specific protective antibodies, precipitins, and agglutinins.

The results of the protective action in mice are given in Table VI. The results in Table VI show that the sera from the immunized rabbits protected mice against only 100 M.L.D. of Type I pneumococci. Although it must be admitted that they do not show conclusively that the new polysaccharide did stimulate antigenic response in rabbits, yet mice receiving sera from the immunized rabbits were more resistant to the fatal infection of Type I pneumococci. It was also found that rabbits immunized with one-tenth or one-hundredth of the above amount, i.e. 0.9 mg. or 0.09 mg. respectively, gave sera which showed no protective action.

Precipitin and agglutination reactions were also performed with the immunized rabbit sera, but no observable reaction could be detected in either instance. This is not surprising since the protection test showed the presence of only a very small amount of protective antibody.

Treatment with Acids and Base.—The effect of hydrogen ion concentration on the precipitative activity and antigenicity of our polysaccharide in mice and rabbits was studied. When a 1:1000 solution of the new polysaccharide was heated for $\frac{1}{2}$ hour at the boiling temperature of water in various acid solutions, such as 0.5 N acetic acid, 0.05 N HCl, 0.2 N HCl, the qualitative precipitin titre was not decreased. The product failed to react with the immune rabbit serum absorbed with the acetyl polysaccharide, but it still precipitated the immune rabbit serum absorbed with the deacetylated polysaccharide of Avery and Heidelberger.

The results are also shown in Table VII. This fact together with the observation that the antigenicity of the polysaccharide is not lost by the acid treatment (see Table VIII) suggests that the acetylated polysaccharide may be the hydrolytic product of our polysaccharide. The chemical nature of the hydrolyzed group will be studied later.

TABLE VI

Protection of Mice against Fatal Infection with Type I Pneumococci, by Rabbit Sera Immunized with the New Polysaccharide

Type I culture	Sera from rabbits						Virulence control
	1	2	3	4	5	6	
<i>cc.</i>							
10^{-4}	D 44 D 44	D 40 D 30	D 44 D 48	D 40 D 48	D 48 D 48	D 50 D 48	— —
10^{-5}	S D 72	S S	S D 76	D 80 D 76	D 72 S	D 72 D 80	— —
10^{-6}	S S	S S	S S	S S	S S	S S	D 44 D 40
10^{-7}	— —	— —	— —	— —	— —	— —	D 40 D 42
10^{-8}	— —	— —	— —	— —	— —	— —	D 40 D 30

Mice weighing 15 to 20 gm. were used. Each mouse received 0.2 cc. serum and 0.5 cc. culture. Sera from these six rabbits before immunization showed no protection in mice against a fatal dose of Pneumococcus Type I.

All mice used for the experiment reported in Table VIII received three doses of 0.5 cc. of 1:million dilution of the original, or acid-treated polysaccharide. The immunizing procedure was identical with that previously given.

TABLE VII

Precipitin Reaction of the Polysaccharide after the Treatment with 0.500 N Acetic Acid

Serum	Final dilution of the polysaccharide hydrolyzed by acids				
	1:10,000	1:40,000	1:160,000	1:640,000	1:2,560,000
Unabsorbed	++++	++++	++++	++	+
Absorbed with the acetyl polysaccharide	—	—	—	—	—
Absorbed with the deacetylated polysaccharide	+++±	++	+	±	—

TABLE VIII

Antigenic Action in Mice of the Newly Prepared Polysaccharide before and after Acid Treatments

Dilution of pneumococcus culture (Type I)	Normal mice		Mice immunized with the newly prepared polysaccharide		Mice immunized with the acid-treated polysaccharide	
cc.						
10 ⁻⁴	—	—	S	S	S	S
10 ⁻⁵	D 30	D 48	S	S	S	S
10 ⁻⁶	D 30	D 48	S	S	S	S
10 ⁻⁷	D 30	D 49	—	—	—	—
10 ⁻⁸	D 30	D 30	—	—	—	—

S = survived. D followed by a number = death occurred at the number of hours. — = not done.

TABLE IX

Precipitin Reaction of the Polysaccharide after the Treatment with 0.05 N NaOH

Serum	Final dilution of the polysaccharide hydrolyzed by alkali				
	1:10,000	1:40,000	1:160,000	1:640,000	1:2,560,000
Unabsorbed	++++	++++	+++	++	+
Absorbed with acetyl polysaccharide	—	—	—	—	—
Absorbed with deacetylated polysaccharide	+++±	++	+	±	—

The effect of the hydroxyl ion was also studied. As shown before, the antigenicity was not destroyed when the polysaccharide was heated at 100°C. for 30 minutes in 0.05 N NaOH solution. The

precipitative activity with the immune rabbit serum absorbed with the acetyl polysaccharide was destroyed. However, it could still react with the unabsorbed immune serum and with serum absorbed with the deacetylated polysaccharide. The results are recorded in Table IX.

The effect of acid on the antigenicity of our polysaccharide in rabbits was also studied. It was found that the polysaccharide obtained after the acid treatment was no longer antigenic, for the sera from three rabbits immunized with the acid-treated polysaccharide afforded no protection on mice against a virulent strain of Type I pneumococci. The result of the protective action of the immunized rabbit sera is given in Table X.

SUMMARY

A type specific polysaccharide has been isolated from the autolyzed broth of Type I *Pneumococcus* by a modified Avery and Goebel's method.

The newly prepared polysaccharide reacts with the homologous immune rabbit serum which has been completely absorbed with the acetyl polysaccharide of Avery and Goebel.

The newly prepared polysaccharide produces passive immunity in mice and rats and possibly in rabbits.

The antigenicity is not lost on boiling in acid or alkaline medium, but the precipitative activity is decreased.

In conclusion, it has been shown that the polysaccharide from Type I *Pneumococcus*, as isolated by a slight modification of Avery and Goebel's method, is a more complete antigen.

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LIPIDS AND IMMUNOLOGICAL REACTIONS

IV. THE LIPID PATTERNS OF SPECIFIC PRECIPITATES FROM TYPE I ANTIPNEUMOCOCCUS SERA

BY FRANK L. HORSFALL, JR., M.D., AND KENNETH GOODNER, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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It has been shown (1) that, after the extraction of lipids, antipneumococcus horse serum fails to give type-specific agglutination or precipitation, and with antipneumococcus rabbit serum these reactions are almost entirely eliminated. If these extractive procedures are carried out in such a manner as to avoid denaturation of proteins the removal of the lipids in no way interferes with the fundamental capacity of the antibody to combine with the specific antigen; lipid extraction merely affects the secondary *in vitro* phenomena of immunological reactions (1, 2).

A tentative hypothesis formulated to explain these results held that antipneumococcus antibodies might be lipo-protein complexes. This theory found some support in the fact that the original agglutinating and precipitating properties of the lipid-extracted immune sera could be restored by the addition of certain purified phosphatides, although in this restoration there was a curious selectivity dependent upon the species origin of the antibody. Thus the "essential" phosphatide for antipneumococcus horse serum is *lecithin*, while that for antipneumococcus rabbit serum is *cephalin* (1).

The more obvious approach to the establishment of this thesis would be through the study of the antibody isolated in a completely pure form. Since the absolute isolation of antibodies has not yet been accomplished, a second and somewhat indirect approach was adopted. Specific immune precipitates were obtained by adding the pneumococcus capsular polysaccharide to antipneumococcus sera. These precipitates might have been expected, from the general theories of antigen-antibody reactions, to contain only the carbohydrate and the antibody. It was found, however, that these precipitates also contained lipid, the amounts varying from 4 to 51 per cent of the total mass of the precipitate (3). This finding was

unexpected in still another sense, for it had been determined in the earlier work (1) that the amounts of phosphatide necessary to restore the *in vitro* properties of extracted antipneumococcus sera were very small (approximately 0.025 mg. per cc.) and thus could not account for the relatively large amounts of lipid in the immune precipitates. As these studies progressed it became increasingly apparent that this large quantity of lipid was, for the most part, present as a result of adsorption, since the amount in the precipitate seemed to be a function of the lipid concentration of the reacting mixture (3). In spite of this fact and even though the probable amount of the essential lipids would be too small to estimate by any of the present chemical methods, it seemed important to determine the nature and amounts of the various lipids in the immune precipitates, particularly with reference to the concentration of these lipids in the immune sera.

In this paper are presented quantitative data determined by the microchemical study of Type I antipneumococcus horse and rabbit sera and of the specific precipitates prepared from them. From these data total lipid patterns have been calculated.

Methods

Two separate lots of unconcentrated and monovalent Type I antipneumococcus rabbit serum and one lot of Type I antipneumococcus horse serum have been used as antibody source. The sera were first filtered through a Berkefeld V and were then whirled at 0°C. for 30 minutes in the angle centrifuge to remove any particulate matter. As the specific precipitant, the acetyl capsular polysaccharide of *Pneumococcus* Type I was used. Precipitates were prepared and washed according to the method of Heidelberger and Kendall (4). The analysis of total nitrogen in the precipitates was carried out in a manner identical to that previously described (3), and lipids were extracted from the precipitates by the technique described in the same paper. Lipid fractions were analyzed by the methods of Kirk, Page, and Van Slyke (5), each determination being made on two aliquots of the extracts. The figures given in Table I represent the mean value of duplicate determinations in each instance. The precipitates were analyzed for total nitrogen, lipid carbon, lipid nitrogen, lipid amino nitrogen, lipid phosphorus, total cholesterol, and free cholesterol. Total lipid, total phosphatide, phosphatide carbon, free cholesterol carbon, esterified cholesterol, cholesterol esters, cholesterol ester carbon, neutral fat carbon, and neutral fat have been calculated from these data according to the various equations of Page, Kirk, Lewis, Thompson, and Van Slyke (6). In Table I of the present paper, these equations are designated by the numbers (*i.e.*, 1 to 7) used by these authors.

In order to obtain precipitates with a sufficient lipid content to permit of adequate analyses, it was necessary to use a considerable quantity of antisera in their preparation. The values given in Table I were found in precipitates prepared in the equivalence zone from 3.0 cc. of antiserum. Other precipitates were pre-

pared under the same conditions from rabbit antiserum by the addition of an equivalent amount of capsular polysaccharide to duplicate 15.0 cc. quantities of serum. In addition, a large amount (0.45 gm.) of specific precipitate¹ which had been prepared from polyvalent Type I and Type II antipneumococcus horse serum by the addition of Type II capsular polysaccharide was divided into equal portions and was analyzed in duplicate for the various lipid fractions. The figures given in Table I are, for the sake of comparison, expressed in milligrams per gram of that part of the total precipitate derived from the immune serum, that is, milligrams per gram of the sum of corrected protein nitrogen multiplied by the factor 6.25 and total lipid carbon multiplied by the factor 1.3 (3). For the antisera themselves the various values are expressed in milligrams per 100 cc. of serum.

Lipid Patterns of Specific Precipitates from Type I Antipneumococcus Horse and Rabbit Sera

Specific precipitates were prepared in the equivalence zone from both horse and rabbit antipneumococcus sera. These were analyzed by gasometric micro methods in order that complete lipid patterns could be calculated. The sera themselves were also analyzed by identical methods so that the lipid patterns both of the sera and of the precipitates could be compared. Directly determined analytical data upon these sera and the precipitates from them, as well as those values calculated from these figures, are presented in Table I.

It will be noted that there was a striking difference between the quantity of lipid amino nitrogen in the precipitate from horse antiserum as compared with that in the precipitate from rabbit antiserum. Of the total lipid nitrogen found in the former, 54.8 per cent was in the amino form, whereas no significant amount of lipid amino nitrogen was detected in the latter. This is in sharp contrast to the almost identical values for lipid phosphorus and indicates a definite qualitative difference in the phosphatides of the two precipitates. That this qualitative difference in the phosphatides of the antisera is demonstrated by the values for total lipid nitrogen, lipid amino nitrogen, and lipid phosphorus in the latter. Of the total lipid nitrogen in the horse antiserum, 35.7 per cent was in the amino form, whereas of the rabbit antiserum lipid nitrogen, 31.1 per cent was amino nitrogen. Except

¹ This precipitate was kindly prepared by Dr. M. Heidelberger.

for this sharp qualitative difference, there was a considerable similarity between the lipid patterns not only of the precipitates but also of the sera themselves. The relationships between the various fractions comprising the total lipid patterns of these antisera and precipitates are shown graphically in Text-fig. 1.

TABLE I

Lipid Patterns of Type I Antipneumococcus Horse and Rabbit Sera and of Specific Precipitates Derived Therefrom

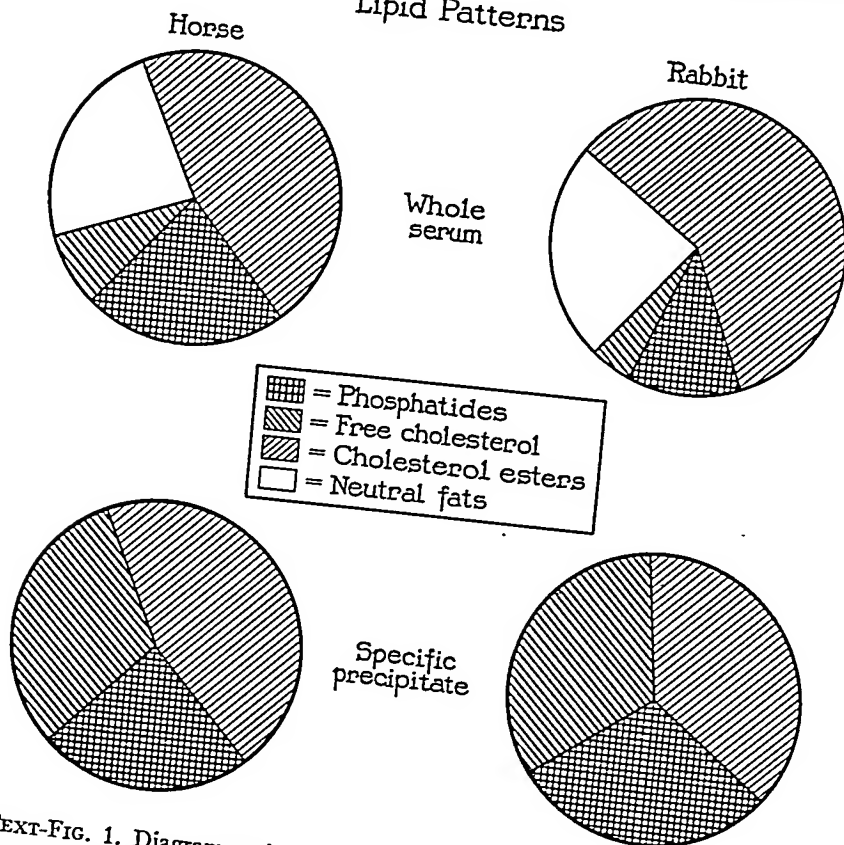
Substance	Method	Type I antipneumococcus serum				Specific precipitates			
		Horse		Rabbit		Horse antiserum		Rabbit antiserum	
		Mg. per 100 cc.	Per cent of total lipid	Mg. per 100 cc.	Per cent of total lipid	Mg. per gm.	Per cent of total lipid	Mg. per gm.	Per cent of total lipid
Total nitrogen.....	Determined					150.2		150.8	
Lipid carbon.....	Determined	194.2		173.7		63.0		52.1	
Total lipid.....	Equation 7	252.5		225.9		81.9		67.7	
Lipid nitrogen.....	Determined	5.41		2.48		3.08		1.54	
Protein nitrogen.....	Calculated					147.1		149.2	
Lipid amino nitrogen.	Determined	1.93		0.77		1.69		0.08*	
Lipid phosphorus....	Determined	2.42		1.15		0.82		0.85	
Phosphatide.....	Equation 1	56.9	22.5	27.0	12.3	19.4	23.9	20.0	29.8
Phosphatide carbon..	Calculated	37.5		17.8		12.8		13.2	
Total cholesterol.....	Determined	87.9		87.1		46.8		37.1	
Free cholesterol.....	Determined	20.6	8.1	10.3	4.7	25.3	31.1	22.3	33.1
Free cholesterol carbon.....	Calculated	17.2		8.7		21.2		18.7	
Esterified cholesterol.	Equation 2	67.3		76.7		21.6		14.8	
Cholesterol esters....	Equation 3	115.5	45.7	129.8	59.1	36.4	44.9	25.0	37.2
Cholesterol ester carbon.....	Calculated	94.3		107.5		30.2		20.7	
Neutral fat carbon...	Equation 4	45.1		39.8		0.0		0.0	
Neutral fat.....	Equation 5	59.5	23.5	52.5	23.9	0.0	0.0	0.0	0.0
Total lipid.....	Equation 6	252.5		219.7		81.1		67.3	

* Quantity not significant.

In order to confirm these results complete lipid patterns have been determined on four separate precipitates from the two lots of Type I antipneumococcus rabbit sera. In these precipitates lipid amino nitrogen was found to account for an average of but 5.62 per cent of

the lipid nitrogen, with a range of 4.32 to 7.44 per cent. Lipid patterns have also been determined on four separate precipitates from the two lots of antipneumococcus horse sera; two from Type I antiserum and two from a mixed Type I and Type II serum. In these instances

Lipid Patterns



TEXT-FIG. 1. Diagrammatic representations of the lipid patterns of Type I antipneumococcus horse and rabbit sera and of specific precipitates derived therefrom. The differences in the phosphatide fractions are fully described in the text.

and amino nitrogen formed an average of 54.68 per cent of the lipid nitrogen, with a range of 54.18 to 55.40 per cent. In the light of these observations, the difference noted in the phosphatide fraction of the lipids in specific precipitates assumes definite

for this sharp qualitative difference, there was a considerable similarity between the lipid patterns not only of the precipitates but also of the sera themselves. The relationships between the various fractions comprising the total lipid patterns of these antisera and precipitates are shown graphically in Text-fig. 1.

TABLE I

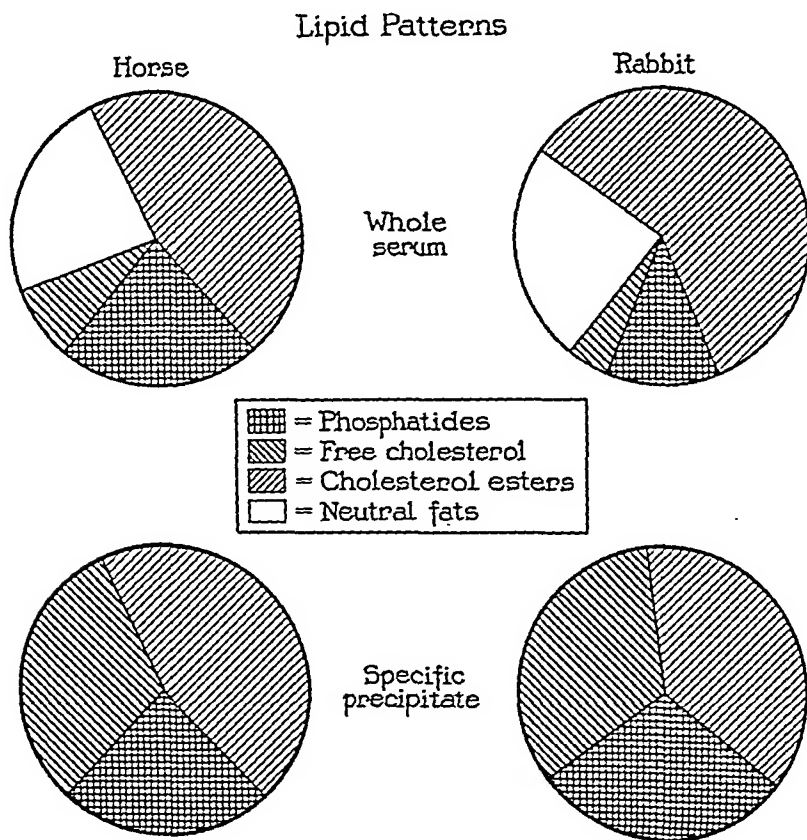
Lipid Patterns of Type I Antipneumococcus Horse and Rabbit Sera and of Specific Precipitates Derived Therefrom

Substance	Method	Type I antipneumococcus serum				Specific precipitates		
		Horse		Rabbit		Horse antiserum		Rabbit antiserum
		Mg. per 100 cc.	Per cent of total lipid	Mg. per 100 cc.	Per cent of total lipid	Mg. per gm.	Per cent of total lipid	Mg. per gm.
Total nitrogen.....	Determined					150.2		150.8
Lipid carbon.....	Determined	194.2		173.7		63.0		52.1
Total lipid.....	Equation 7	252.5		225.9		81.9		67.7
Lipid nitrogen.....	Determined	5.41		2.48		3.08		1.54
Protein nitrogen.....	Calculated					147.1		149.2
Lipid amino nitrogen.....	Determined	1.93		0.77		1.69		0.08*
Lipid phosphorus....	Determined	2.42		1.15		0.82		0.85
Phosphatide.....	Equation 1	56.9	22.5	27.0	12.3	19.4	23.9	20.0
Phosphatide carbon..	Calculated	37.5		17.8		12.8		13.2
Total cholesterol.....	Determined	87.9		87.1		46.8		37.1
Free cholesterol.....	Determined	20.6	8.1	10.3	4.7	25.3	31.1	22.3
Free cholesterol carbon.....	Calculated	17.2		8.7		21.2		18.7
Esterified cholesterol.....	Equation 2	67.3		76.7		21.6		14.8
Cholesterol esters....	Equation 3	115.5	45.7	129.8	59.1	36.4	44.9	25.0
Cholesterol ester carbon.....	Calculated	94.3		107.5		30.2		20.7
Neutral fat carbon....	Equation 4	45.1		39.8		0.0		0.0
Neutral fat.....	Equation 5	59.5	23.5	52.5	23.9	0.0	0.0	0.0
Total lipid.....	Equation 6	252.5		219.7		81.1		67.3

* Quantity not significant.

In order to confirm these results complete lipid patterns have been determined on four separate precipitates from the two lots of Type I antipneumococcus rabbit sera. In these precipitates lipid amino nitrogen was found to account for an average of but 5.62

the lipid nitrogen, with a range of 4.32 to 7.44 per cent. Lipid patterns have also been determined on four separate precipitates from the two lots of antipneumococcus horse sera; two from Type I antiserum and two from a mixed Type I and Type II serum. In these instances



TEXT-FIG. 1. Diagrammatic representations of the lipid patterns of Type I antipneumococcus horse and rabbit sera and of specific precipitates derived therefrom. The differences in the phosphatide fractions are fully described in the text.

lipid amino nitrogen formed an average of 54.68 per cent of the lipid nitrogen, with a range of 54.18 to 55.40 per cent.

In the light of these observations, the difference noted in the phosphatide fraction of the lipids in specific precipitates assumes definite

significance. Since both rabbit and horse antisera have been shown to contain amino and non-amino phosphatides in similar amounts, the very small amount of amino phosphatide in the specific precipitate from rabbit antiserum and the large amount of amino phosphatide in the specific precipitate from horse antiserum suggests the possibility that a selective adsorption of phosphatide by the forming precipitates occurs.

Certain additional studies have been made upon the effect of the addition of purified phosphatides to horse antipneumococcus serum prior to the formation of specific precipitates. The results of these studies are not entirely conclusive, since it is extremely difficult to add lipids in any state other than an unnatural colloidal suspension, but it may be stated that evidence has been obtained which indicates that the presence of added lecithin or cephalin in the reacting mixture causes a marked reduction in both the total and free cholesterol of the precipitates. This decrease in cholesterol in the precipitates is more pronounced in the presence of added lecithin than in the presence of added cephalin.

DISCUSSION

The data presented indicate that, although the specific precipitates from Type I antipneumococcus horse and rabbit sera contain relatively similar amounts of total lipid, the nature of the various separate lipids comprising this total is different for the two species. This difference in the lipid patterns of the precipitates appears to lie almost entirely in the phosphatide fraction and is only detected when lipid nitrogen and lipid amino nitrogen analyses are compared. It then becomes evident that, although 54.6 per cent of the lipid nitrogen of precipitates from horse antisera is in the amino form, only 5.6 per cent of the lipid nitrogen in precipitates from rabbit antisera is amino nitrogen.

Since it has been shown that the relationships between lipid phosphorus, lipid nitrogen, and lipid amino nitrogen are almost identical in the antisera themselves, it is not possible to explain the observed differences in the phosphatides of the precipitates simply on the basis that they are a reflection of the lipid patterns of the antisera.

Of those phosphatides known to occur in sera, lecithin and cephalin form much the greater part. The former contains no amino nitrogen, and of the nitrogen present in the latter all is in the amino form. Although accurate micro methods have not yet been developed which allow of the direct estimation of either lecithin

or cephalin in small amounts, it is possible to gain an approximation of their quantity by indirect means. This involves calculations based upon lipid phosphorus, lipid nitrogen, and lipid amino nitrogen. When, as in the case of the lipid fraction of the specific precipitate from rabbit antisera, almost insignificant quantities of lipid amino nitrogen are found and appreciable amounts of both lipid phosphorus and lipid nitrogen are determined, it becomes obvious that cephalin, an amino nitrogen-containing phosphatide, is present in amounts so small as almost to escape detection by the methods used. In order to account for both the lipid phosphorus and lipid nitrogen found it is necessary to assume that in this instance these form parts of a non-amino nitrogen-containing phosphatide. Since lecithin fulfills these requirements and is known to constitute a large portion of the phosphatides of serum, it is logical to suppose that lecithin is present and comprises much the larger proportion of the phosphatide in the specific precipitate from rabbit antiserum.

On the other hand, when, as in the case of the specific precipitates from horse antisera, as much as 54.6 per cent of the total lipid nitrogen is in the amino form, and when this quantity of amino nitrogen is considerably in excess of the theoretical amount necessary to give an atomic ratio of 1 with the lipid phosphorus found, it becomes apparent that the larger part of the phosphatide present in the precipitate contains amino nitrogen. Since cephalin contains both amino nitrogen and phosphorus, and is known to be present in serum, it is fair to consider that the phosphatide fraction in the specific precipitates from horse antisera is largely cephalin.

Finally, it may be suggested that since the lipid patterns of specific precipitates from horse and rabbit antisera are quite different as regards the nature of the phosphatide fraction, although the lipid patterns of the antisera themselves are very similar, selective adsorption of phosphatides by the two different precipitates may account for the observed dissimilarity. It appears that from serum containing both lecithin and cephalin, a forming precipitate resulting from the union of the horse antibody and the homologous capsular polysaccharide carries down an amino nitrogen-containing phosphatide which is probably for the most part cephalin, while under the same circumstances the rabbit antibody-polysaccharide complex carries down a non-amino nitrogen-containing phosphatide which seems to be largely lecithin.

These findings present a curious paradox. It has been shown that lecithin restores the *in vitro* properties of horse antiserum extracted by

lipid solvents, and that cephalin acts similarly in the case of rabbit antiserum, whereas cephalin will not affect extracted horse serum nor lecithin extracted rabbit serum (1). Because of this finding these two phosphatides have, in a descriptive sense, been termed essential and complex in horse antiserum, and that cephalin may be similarly placed in rabbit antiserum. The analyses of specific precipitates from horse and rabbit immune sera have shown what appears at first glance to be exactly the opposite, for the phosphatide in the precipitate from horse antiserum is almost entirely cephalin, while that in the rabbit precipitate is very largely lecithin. It must be emphasized, however, that the amount of so called essential phosphatide is very minute, as little as 0.025 mg. per cc. of extracted serum being sufficient to restore *in vitro* properties. Of the various precipitates analyzed, the largest was prepared from 20.0 cc. of antiserum, and from theoretical considerations would therefore contain but 0.50 mg. of essential phosphatide, at most. In the methods used this quantity of either lecithin or cephalin would give but 0.00093 mg. and 0.0026 mg. of directly determinable nitrogen and phosphorus respectively, amounts which are too minute to permit of accurate estimation.

Although these findings do not contribute to the solution of the important question as to whether the antibody is a phosphatide-globulin complex, they do demonstrate that antigen-antibody aggregates possess curious and selective properties dependent upon the species derivation of the antibody. It is suggested that these properties may explain certain differences in the various immunological characteristics of these two immune sera (7, 8).

SUMMARY

1. Complete lipid patterns of specific precipitates from horse and rabbit Type I antipneumococcus sera, as well as of the sera themselves, have been determined by gasometric micro methods.
2. The lipid patterns of horse and rabbit antisera are very similar, and as regards the phosphatide fractions are relatively identical.
3. The lipid patterns of specific precipitates from horse and rabbit antisera show one outstanding qualitative difference. The specific precipitate from horse antiserum contains an amino phosphatide.

which is probably cephalin, while that from rabbit antiserum contains a non-amino phosphatide, which is thought to be lecithin.

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A QUANTITATIVE TECHNIQUE FOR PERFORMING PLASMAPHERESIS

By DANIEL MELNICK,* Ph.D., AND GEORGE R. COWGILL, Ph.D.

WITH THE COOPERATION OF ETHEL BURACK,** Ph.D.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven)

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Repeated bleedings of an animal, followed immediately by the reinjection of corpuscular elements of the blood suspended in Locke's solution, results in depletion of the plasma. This procedure, a modification of that used by Morawitz (1), was first employed by Abel, Rowntree and Turner (2), and was called plasmapheresis by these investigators.

This technique was successfully used by Whipple and his associates (3-5) in studying the effects of diet and fasting on the curve of serum protein regeneration. Recently the Whipple group (6-8) has attempted to evaluate dietary factors as specific agents for promoting the formation of new serum protein. However, in these studies (6-8) plasmapheresis does not appear to have been performed quantitatively; judging from the data as reported (7, 8), the blood protein level was not constant but varied from 3.18 to 4.69 per cent. Thus the intensity of the stimulus for serum protein regeneration during each of the dietary periods in all probability was not the same.

In any quantitative approach to the study of the influence of diet upon the regeneration of serum protein it is essential to maintain the basal level of serum protein constant. In our investigation of this problem, plasmapheresis was performed by what we believe to be a highly quantitative method. By daily *preliminary* determinations

*This paper is a report of certain material contained in a dissertation presented by Daniel Melnick in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1936. A part of the expense of this investigation was borne by a grant to George R. Cowgill from the Research Fund of the Yale University School of Medicine.

** Standard Brands Inc. Fellow, 1934-1936.

of the serum protein concentration and weekly *preliminary* determinations of the blood volume, the size of each bleeding was calculated to reduce the serum protein concentration of the dog to 3.5 per cent. To avoid the withdrawal of insignificant quantities of blood, plasmapheresis was conducted only on those days when the serum protein level reached or surpassed 4.2 per cent. During the initial week, when the "reserve serum protein store" of the animal was depleted and the concentration of the blood protein reduced to the basal level, bleedings of one-fourth of the blood volume were performed daily.

The Bleeding Phase

The method usually employed in the clinic for withdrawing blood proved to be unsatisfactory in the present study for several reasons. For our purpose it was desirable to withdraw large volumes of blood accurately to within ± 1 per cent of the calculated amounts. Furthermore, it was essential to bleed the experimental animals with the least possible injury to the femoral artery so that on the succeeding days there would be no difficulty in palpating the vessel due to any excessive production of scar tissue or hematomas as a result of leakage at the sites of previous punctures. Therefore, a method, whereby the blood could be collected rapidly through a needle of relatively small bore with the artery having been entered but once, was sought. Finally, the tendency exhibited by the dogs' blood to clot rapidly before the desired amounts were obtained, prevented quantitative blood withdrawals in our preliminary trials. This occurred frequently in spite of coating the inner surface of the rubber tubing with paraffin and even after preliminary rinsing of all the tubing with the anticoagulant solution.

For the present study a special apparatus and technique were finally devised, allowing one to overcome the difficulties enumerated above. The method permits the rapid removal of large quantities of blood aseptically, accurately, with a minimum of hemolysis and without any danger of clot formation during the process. A photograph of the apparatus is shown in Fig. 1.

The 500 cc. graduate (F) is calibrated and correction made for the volume occupied by the stirrer (G). 1 per cent of the volume read on the graduate is due

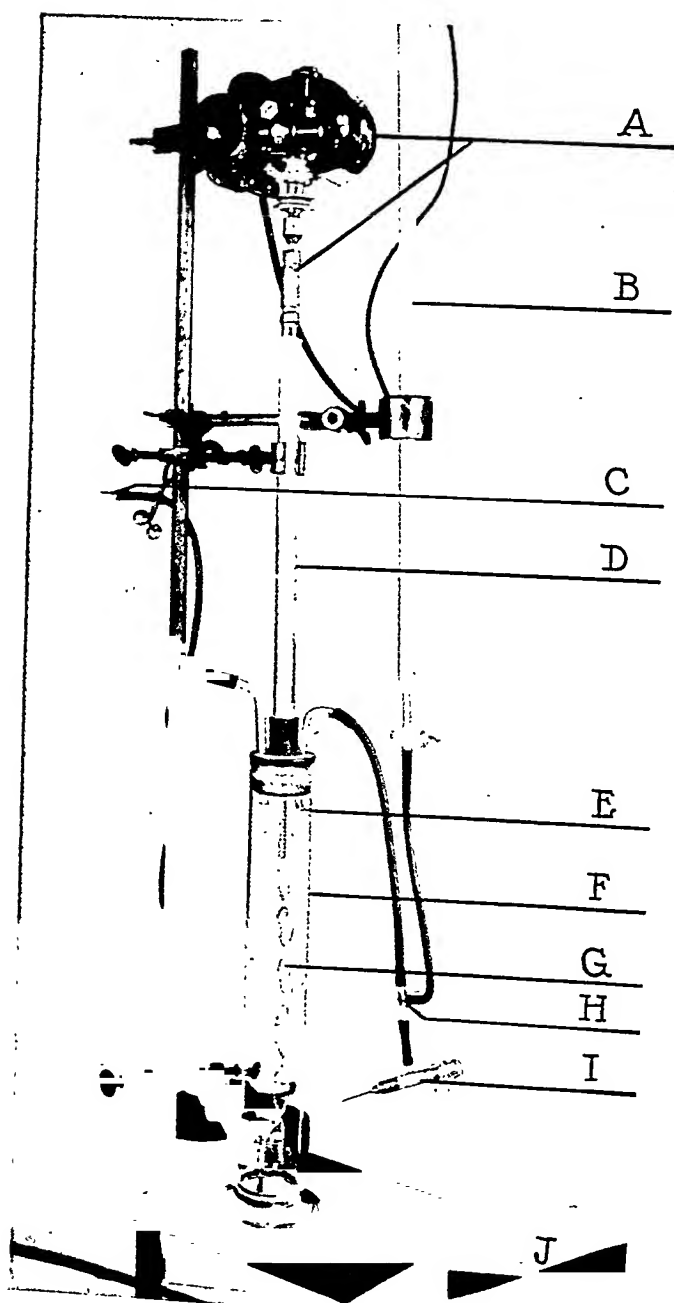


FIG. 1. The bleeding apparatus.

to the presence of the stirrer. The motor driven stirrer (*A*) rotates at about 150 to 200 revolutions per minute. This feature not only permits the system to be relatively compact, giving less play of rubber tubing, but also enables two individuals to conduct a bleeding in a highly efficient manner. The minimal agitation produced by the rotating stirrer, the spiral form of the active stirrer (*G*) and the construction of the inlet tube (*E*), causing the blood to flow gently down the side of the graduate, reduce hemolysis to a minimum. Determinations of the extent of hemolysis produced during this stage of plasmapheresis were carried out as a preliminary step in a later study. The value of 2.5 mg. of hemoglobin per 100 cc. of citrated plasma appears to be a valid expression of the hemolytic effects of this initial stage of plasmapheresis. The mercury seal (*D*) allows efficient stirring with the system under a negative pressure of 230 to 240 mm. of mercury. The negative pressure is maintained by suction at the mouthpiece (*C*). The burette (*B*) contains the anticoagulant, a 3 per cent solution of anhydrous sodium citrate being used; the volume of the citrate solution used is regulated so that the final concentration will be 0.3 per cent, or a 1 to 10 dilution. At the three-way tube (*H*) the anticoagulant comes in contact with the blood. The arm of the tube leading to the burette has a capillary bore so that the greatest part of the negative pressure in the system is exerted on the incoming blood. To the three-way syringe (*I*), which is of the B-D Yale Kaufman, Luer-lok type, is attached a No. 19 platinum needle. This needle has proven to be very satisfactory inasmuch as its bore is equivalent to that of a No. 18 steel needle.¹ Due to the weight of the motor the apparatus is unstable, but accidents due to this instability are easily prevented by clamping (*J*) the supporting stand to a firm surface, the table, for example.

The apparatus is rendered aseptic by consecutive washings with a mercuric cyanide solution, sterile water, alcohol and ether. To dry the system completely, air is sucked through a concentrated sulfuric acid bath, in place of the syringe, and through a sterile cotton plug inserted in the mouth of the burette, the stop-cock being open. The suction from a house line is applied at the mouthpiece (*C*). Bacteriological analyses indicated that this technique does indeed give satisfactory asepsis.

From 3 to 5 cc. of the calculated amount of the sterile 3 per cent sodium citrate solution is first sucked through the needle into the graduate; the remaining citrate is poured into the burette. The needle is inserted into the femoral artery, and the plunger of the syringe withdrawn beyond the side arm. The system is maintained under negative pressure. Simultaneously the citrate is added slowly from the burette at a rate which roughly approximates 1/10 of that of the blood flow. The stirring completely mixes the blood with the anticoagulant. About 5 cc. of the citrate is left in the burette. When the citrated blood has reached a volume 10 cc. below the desired amount, the needle is withdrawn. The amount of blood in the syringe and rubber tubing together with the remaining citrate used to flush

¹ We are indebted to Dr. Daniel C. Darrow of the Department of Pediatrics for this suggestion.

out the tubing brings the volume of citrated blood to that desired. 400 cc. of blood may be collected easily within a 3 to 5 minute interval, the needle having been placed in the artery only once. The operator handles the syringe and nothing else. His assistant can easily operate the apparatus, pet the dog and signal when to withdraw the needle from the artery. It is very convenient to have the graduate marked with crayon at the reading corresponding to the desired blood volume and the mark 10 cc. below this. With a little experience, the volumes of blood obtained are accurate and consistent.

The method for calculating the volume of blood to be removed is exceedingly simple. The only assumption involved is that the plasma volume and the serum volume are equal. Any error arising from the volume occupied by the fibrinogen must be exceedingly small, being undoubtedly less than 1 per cent. By elementary algebra it is easily shown that the hematocrit, or the volume per cent of blood representing the plasma or serum, does not enter into the calculation.

If we let

x = the blood volume in cubic centimeters,

y = the fraction of the blood represented by the plasma,

xy = the plasma volume in cubic centimeters,

z = the serum protein concentration in grams per cent as determined daily,

3.5 = the serum protein level in grams per cent to which the concentration is to be lowered each day that bleeding occurs,

$z - 3.5$ = the serum protein increment to be removed;

then

$$\frac{z - 3.5}{z} \cdot xy = \text{plasma volume in cubic centimeters to be removed,}$$

and

$$\frac{\frac{z - 3.5}{z} \cdot xy}{y} = \text{blood volume in cubic centimeters to be removed,}$$

or

$$\frac{z - 3.5}{z} \cdot x = \text{blood volume in cubic centimeters to be removed.}$$

The formula last given is the one to be used. A representative experiment will serve to illustrate all the details in the calculations.

Dog 4. (17.16 kilos) June 3, 1935. Blood volume 1425 cc. (as determined on June 1, 1935). Serum protein concentration 5.04 per cent.

$5.04 - 3.50 = 1.54$ per cent serum protein increment to be removed.

Then

$$\frac{1.54}{5.04} \cdot 1425 = 437 \text{ cc. of blood to be removed,}$$

$$\text{and } 437 \cdot 1/9 = \frac{49 \text{ cc. citrate to be added,}}{486 \text{ cc. total of citrated blood,}}$$

$$\text{and } \frac{5 \text{ cc. volume occupied by stirrer, or about 1 per cent,}}{491 \text{ cc. reading on the graduate.}}$$

therefore

The graduates were always marked with a crayon to the nearest 5 cc. mark; in the above case, 490 cc. In Table I are listed representative bleedings, as obtained experimentally and as calculated, to show the extent of agreement. These were obtained with dog 4. Using our technique, the average deviation is easily kept within ± 1 per cent; in the series listed in Table I it is ± 0.6 per cent. The omissions on June 2, 9, 16, and 23 were due to the fact that no bleedings were conducted on Sundays.

For the most part the volumes of blood withdrawn were not so large as to cause physiological disturbances (6, 9, 10), because subsequent to the hemorrhage a red cell suspension was injected immediately.

Preparation of Cells for Reinjection

Plasmapheresis necessarily involves the reinjection of the corpuscular elements of the blood. The details of the preparation of the cells in accordance with our technique are given below.

The citrated blood is transferred to calibrated 100 cc. centrifuge bottles which had previously been graduated accurately to the nearest cubic centimeter. These containers are sterilized either by the same procedure as that described above or by having cleaning solution (potassium dichromate in concentrated sulfuric acid) replace the mercuric cyanide solution. The rubber stoppers are so cut as to retain a flange extending about 6 mm. beyond the mouth of the bottle. This feature prevents the stoppers from being driven into the tubes during centrifugation and also aids in the asepsis. A small glass plunger, running through the center of the stopper, also with a flange, permits the complete filling and stoppering of the bottles with a minimal production of pressure. The stoppers are sterilized in boiling water. These and the tubes are then kept for 10 minutes in an oven at 105°C. to drive off the last traces of alcohol and ether. The mouths of these bottles are always swabbed with alcohol whenever they are opened or stoppered. Centrifugation for 1 hour at 2100 R.P.M. is satisfactory for the determination of the cell volume. After correcting for the citrate present, the hematocrit is calculated. Following the removal of the citrated plasma by suction, physiological saline is added in approximately equal volume. The saline suspension of cells is then stored in the refrigerator at 5-8°C. until the next plasmapheresis. The

longest time interval, during which we have stored these cell suspensions, has been 4 days; on injection these proved to be utilized satisfactorily. After a serum protein determination, which indicates the necessity for a plasmapheresis in order to maintain the basal level constant, the saline suspension of cells is centrifuged (1500 R.P.M.) for 1 hour, the saline removed by suction to be replaced by an approximately equivalent volume of modified Locke's solution (5). The Locke's solution is made daily from water freshly distilled (11) from pyrex glassware (12) and contains 0.900 per cent sodium chloride, 0.042 per cent potassium chloride and 0.020 per cent sodium bicarbonate.²

TABLE I
Quantitative Plasmapheresis: Bleeding Phase

Date	Bleeding		Deviation
	Calculated	Experimental	
1935	cc.	cc.	per cent
May 31	346	347	+0.3
June 1	335	334	-0.3
3	437	436	-0.2
4	392	392	±0.0
5	286	295	+3.2
6	339	340	+0.3
7	238	243	+2.0
8	358	356	-0.6
10	451	451	±0.0
11	346	352	+1.7
12	298	299	+0.3
13	364	365	+0.3
14	360	361	+0.3
15	333	334	+0.3
17	473	472	-0.2
18	394	393	-0.3
19	400	400	±0.0
20	438	436	-0.5
21	370	370	±0.0
22	382	383	+0.3
Average deviation.....			±0.6%

Reinjection of the Cells

At the initial plasmapheresis, subsequent to the hemorrhage, a suspension of cells obtained from one of the donor animals is used.

² This solution is easily made by dissolving 9.62 gm. of modified Locke's mixture, composed of 93.55 per cent NaCl, 4.37 per cent KCl and 2.08 per cent NaHCO₃, in 1 liter of freshly distilled water.

Thereafter the cells removed in the preceding plasmapheresis are reinjected immediately after the bleedings. Smith, Belt and Whipple (5) had conducted plasmapheresis in which the injection was simultaneous with the hemorrhage. By such a procedure a considerable portion of the bleeding is due to the passive withdrawal of the injected cell suspension. Later (6-8) this was modified so that the injection phase followed immediately after the bleeding. In the present study such a procedure has been followed.

The apparatus employed for the reinjection of the cells is shown in Fig. 2.

The tube (*H*) is filled with the Locke's solution, care being taken to eliminate all air bubbles. The suspension of cells is then filtered through 8 layers of sterile gauze into the gravity tube (*F*). The outside jacket (*E*) contains water at 40°C. to keep the cell suspension at approximately body temperature. The cells are injected under positive pressure maintained by an atomizer bulb (*A*). Due to the considerable pressure required for the injection of the cells, it is essential to have the stopper of the gravity tube held firmly in place by adhesive tape (*C*). The tube holds only 270 cc. so that the subsequent additions are made through the tube (*B*). The opening of this tube is so constructed that the blood flows gently down the side of the gravity tube with no foaming and the minimum of hemolysis. About 100 cc. of Locke's solution is used to wash down the sides of the gravity tube subsequent to each injection. Thus, there are injected each day 100 cc. of fluid in excess of that withdrawn. On those days, when donors' cells are injected in addition to the homologous cells in order to keep the hematocrit normal, the extra Locke's solution is dispensed with. When these washings have passed the pilot tubes (*I*), the three-way syringe is closed, thus avoiding the possible injection of air bubbles. Most of the cell injections were made with a No. 18 needle inserted into the jugular vein; occasionally the radial and saphenous veins were resorted to. The injection of a cell suspension and extra Locke's solution totaling 500 cc. required approximately 5 to 10 minutes. An animal can easily tolerate this rate of injection (13).

The injection of the suspension of cells is carried out aseptically. The gravity tube, stopper and tubing are sterilized in boiling water in a closed sterilizing dish. The entire system is rinsed with sterile physiological saline solution before the cells are added. In the early experiments a nonabsorbent cotton filter was inserted in the glass tube to which the pressure bulb was attached. This was omitted later because it interfered with the production of an adequate pressure. Undesirable symptoms as a result of this breach of asepsis were never observed in our dogs.

All rubber tubing and stoppers in both sets of apparatus are treated by boiling with $N/2$ sodium hydroxide, rinsing with water, boiling with $N/2$ hydrochloric

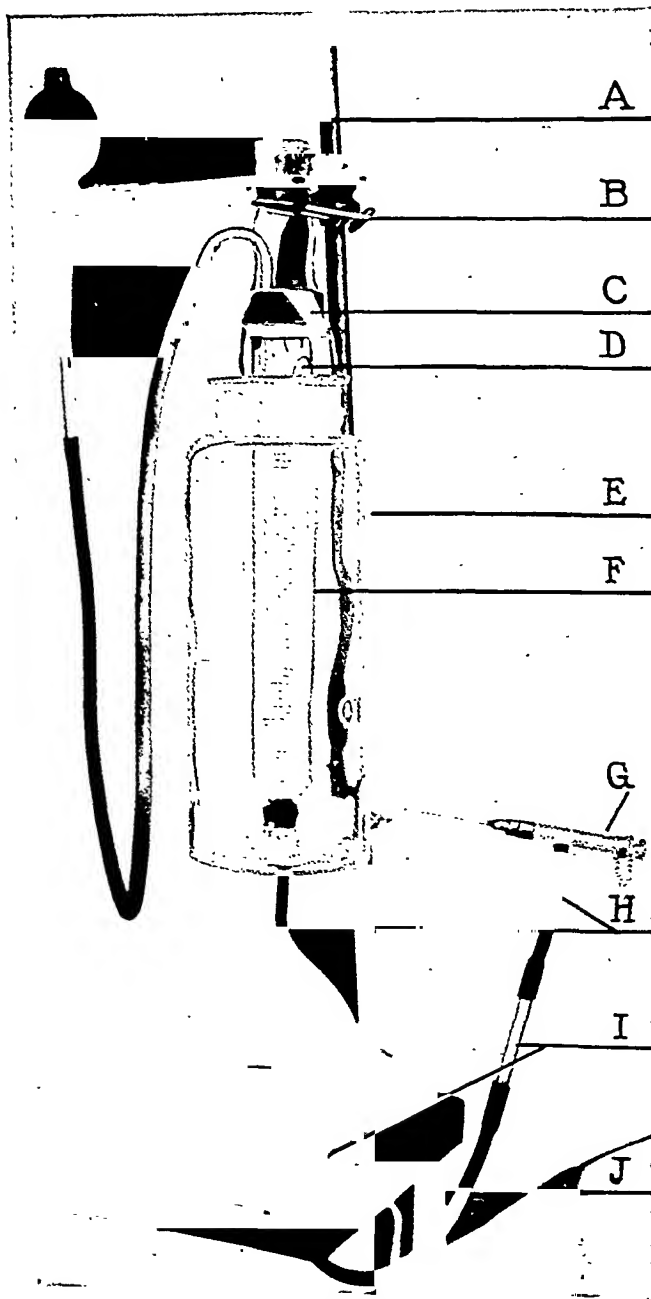


FIG. 2. The cell infusion apparatus.

acid, and a final rinsing with water. Such a preliminary procedure eliminates any toxic reactions (14) which may follow blood transfusions involving the use of apparatus with rubber tubing.

For the determination of the serum protein concentration each day previous to a bleeding, in order to calculate the amount of blood to be withdrawn, a method was sought which would be rapid, reasonably accurate and would require a small amount of blood. The nephelometric method devised by Rona and Kleinmann (15) was found to be suitable. The technique involves the comparison of the turbidities produced by the standard and unknown when an aqueous solution of sodium sulfosalicylate is added to the dilute (1 to 200) serum protein solutions after they had been strongly acidified with hydrochloric acid.

The turbidities produced vary with the concentration of serum protein according to the nephelometric curve (16). The results of more than 400 of our own determinations in duplicate indicate that one may expect simultaneous estimations to agree to within ± 0.5 per cent of the mean.

Rona and Kleinmann (15) report that relatively large concentrations of ammonium sulfate, magnesium sulfate, Ringer's solution, thymol and decomposition products of proteins have only negligible effects as interfering factors in the method. In our own experience it has been found qualitatively that no turbidities are produced when the sulfosalicylic acid is added to solutions of proteose or peptone, obtained as products of peptic digestions. In fact, the reagent may be used as an indicator of the complete removal of acid meta-protein from a weak acid hydrolysate of protein. The precipitation of the protein presumably is not dependent on its large molecular size because solutions of dextrin or starch paste are not affected by the reagent. The only nonprotein substances which are precipitated by the reagent, as far as we know, are plant alkaloids.

The specificity of this method for the determination of the serum protein concentration in dogs subjected to plasmapheresis was confirmed by (a) determining the concentration of serum protein when present in a serum sample and subsequently when dissolved in physiological saline, and (b) observing that the addition of sulfosalicylic acid either to a suspension of the serum lipids in physiological saline or to a solution of the serum crystalloids fails to produce any turbidity.

As final proof of the validity of the technique, the results yielded by this nephelometric procedure were checked against those obtained by standard methods. The average percentage deviation of the nephelometric values from those obtained by Kjeldahl analyses (17, 18) on nine representative samples was ± 4.0 per cent; the percentage deviations varied from $+0.5$ to -5.7 per cent. When compared with the results obtained gravimetrically (19, 20) on five other serum samples the average percentage deviation of the nephelometric values was found to be ± 2.9 per cent, varying from -1.4 to $+5.3$ per cent.

For the estimation of blood volume, the dye method as improved by Hooper, Smith, Belt and Whipple (21) was employed. The exact validity of any method for the determination of the blood volume can, of course, be questioned (22). However, in our studies the same procedure was used throughout, so that any errors inherent in the method were reasonably constant during each of the experimental periods.

SUMMARY

1. A special apparatus and technique are described which permit one to conduct plasmapheresis quantitatively.
2. The validity of the methods employed, for determining serum protein concentration and blood volume as prerequisites for the calculation of the amount of blood to be withdrawn, are discussed.

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THE INFLUENCE OF DIET UPON THE REGENERATION OF SERUM PROTEIN

I. STANDARDIZATION OF EXPERIMENTAL TECHNIQUE

BY DANIEL MELNICK,* PH.D., GEORGE R. COWGILL, PH.D., AND
ETHEL BURACK,** PH.D.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven)

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Using the technique of plasmapheresis, Kerr, Hurwitz and Whipple (1, 2) observed that dogs receiving food regenerate blood plasma protein more rapidly than fasting animals. Later Smith, Belt and Whipple (3) modified the experimental method by reinjecting the cell suspension simultaneously with the bleeding and reported a rapid regeneration within 15 minutes after the hemorrhage. This rate of recovery then decreased, 2 to 7 days being required for return to a normal value. The rapid replacement of serum protein immediately after plasmapheresis was interpreted as indicating a reserve store of this material.

Following the demonstration that diet will promote the regeneration of serum protein from an abnormally low value to the normal, Whipple and collaborators (4-6) have attempted to evaluate dietary factors as specific agents for stimulating this recovery. Plasmapheresis, while the animals were fed a basal diet, was continued 4 to 6 weeks before the dogs exhibited an approximately constant production of plasma proteins. The reduction of the plasma protein level to 4.0 per cent was considered to act as a constant and maximal stimulus for the regeneration of serum protein. Potent dietary factors necessitated larger and more frequent bleedings to maintain the concentration constant at the desired low level, designated as the basal level. Subsequent to the exhaustion of the "reserve serum protein stores,"¹ while subsisting on the basal diets the dogs were able to produce

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** Standard Brands Inc. Fellow, 1934-1936.

¹ This expression, used by Whipple and collaborators as indicative of the amount of serum protein which must be withdrawn before the animal manifests a *constant* output of serum protein under a given basal dietary régime, will be employed without quotation marks in the subsequent discussions.

10 to 30 gm. of new plasma protein each week. The ratio of dietary protein fed per week to the amount of plasma protein removed, over and above the basal output, was termed the potency ratio for that protein (5). Values were reported varying from 2.6 for beef serum protein to 20.8 for kidney protein; the more potent a protein, the smaller the ratio proved to be. These investigations were carried out with what we believe to be numerous uncontrolled variables operating, and for this reason we have reinvestigated this problem.

The prime objective in the present study was to incorporate as part of the technique the use of artificial diets, the compositions of which were carefully controlled.

Examination of the previous work on this problem appears to justify the following comments:

1. The plasma protein level for each dietary period was not as constant as might be desired but varied from 3.18 to 4.69 per cent. In all probability, therefore, the intensity of the stimulus for serum protein regeneration during each of the dietary periods was subject to corresponding variation.

2. The basal diet (4, 5), composed of natural foods, contained 7 per cent of the caloric intake in the form of protein. With such a ration, 4 to 6 weeks were found necessary in order to deplete the body of the reserve store of protein building material. Furthermore, 2 weeks were required to deplete the animals of the "carry overs" from previous dietary periods. In their most recent paper (6) several *basal* diets were employed which were superior to the earlier ration (4) in that they were *less effective* in promoting serum protein regeneration.

3. The diets were composed of natural foods. Each dietary change constituted actually a change in the entire diet, for example, one animal tissue replacing another (5). Subsequently, an attempt was made to deal with the dietary factor quantitatively by superimposing on the basal diet some natural food (6) which was considered the only variable introduced into the experimental régime. This, however, is not consistent with the view that proteins in a heterogeneous mixture can exert supplementary effects upon each other.

4. The amounts of protein ($6.25 \times$ per cent N) fed during each dietary period were extremely variable, ranging from 43 gm. of basal dietary protein to 578 gm. of pancreatic protein per week. In evaluating these dietary changes the potency ratio was assumed to be independent of the amount of protein in the diet. However, it has been shown that the coefficient of utilization of an essential dietary component, in this case the protein (7-9), decreases when an excess of the substance is fed.

5. In the earlier papers (4, 5) the animals were fed *ad libitum* so that the daily food intake was not constant. Thus the dietary factor, as a stimulus for the regeneration of plasma protein, probably varied from day to day. The work to be reported was undertaken before the appearance of the most recent publication in this field (6), in which the basal diets were fed at a constant level of caloric intake.

6. In calculating the potency ratios of the substances tested, Whipple and associates seemed to disregard as of no significance that part of the protein intake which is used to satisfy nitrogen equilibrium. Urinary nitrogen data were obtained for each period, but they proved to be of limited value, serving only to indicate the character of the nitrogen balance. Although 3 to 7 days were observed to be essential for the dog to adapt itself to the altered protein intake, no allowance was made for this adjustment period; instead, the subsequent carry over values were included in the data of the previous experimental period. Such a procedure assumes that any lag in serum protein regeneration during the week when the test protein diet is fed, regardless of the nature of the protein, is completely compensated for by the carry over values.

7. In an investigation of this nature in which a limited number of animals may be employed, each animal should be its own control. In the previous work here under review, 24 dietary factors were studied on nine dogs (4-6). In most cases, it appears that confirmatory evidence was not sought (see Table 6 in Reference 6). However, the potency ratio of one dietary factor is compared with another, although two different dogs may have been employed to arrive at these values, and the basal diets of these two animals may not have been the same.

In 1932, the Chinese investigators Liu, Chu, Wang and Chung (10) in a study of nutritional edema in man (two patients) demonstrated that adequate amounts of dietary protein are beneficial in alleviating the disease. "Nitrogen-free" diets reproduced the edema with concomitant decreases in the plasma protein concentration. Salt and water intake and physical activities were excluded as possible factors in the causation of edema by suitable control of these variables in the studies. The administration of animal proteins at a level of 1 gm. per kilo of body weight (7 per cent of the caloric intake) resulted in a prompt and marked nitrogen gain, plasma protein increase and disappearance of edema. The same results were achieved by feeding twice the above amount of vegetable proteins. A vegetable protein intake equivalent to that of the animal proteins proved to be inadequate.

The interpretation of their findings as indicating that animal proteins are twice as efficient as vegetable proteins in causing a regeneration of serum protein seems erroneous. In their experiments two factors were competing for required dietary protein, namely, the attainment of nitrogen equilibrium for the entire organism and the regeneration of serum protein. The fact that animal proteins are superior to vegetable proteins for the daily metabolism of the normal organism (11), indicates that during comparable feedings of these proteins a greater aliquot in the case of the animal proteins is available for the regeneration of serum protein. Indeed, these investigators (10) have also recorded a superior biological value for their animal protein diets in comparison with those containing vegetable proteins. Moreover, the entire investigation was still further complicated by the fact that the patients were suffering from generalized metabolic disturbances including a marked diarrhea. Thus, in the case of one patient 46.5 per cent of the dietary nitrogen was excreted in the feces; and in the other, 31.2 per cent. The only positive finding obtained by the authors was the successful demonstration that

the most important etiologic agent in nutritional edema is inadequate protein intake.

Plan of Study

In the present investigation attempts have been made to control the many variables described above. Plasmapheresis appears to be the only satisfactory experimental approach to the problem. Thus, in conditions of nutritional edema with a hypoproteinemia, one has no means of evaluating the dietary factors quantitatively, inasmuch as the experimental subject has been exposed to the effects of a prolonged negative nitrogen balance together with an inadequate caloric intake. As a result of this, three processes compete for the available dietary protein: (a) the repair of wasted tissues; (b) the satisfaction of the normal daily maintenance metabolism; (c) the regeneration of serum protein. The modifications of experimental technique employed in the present study are described below and are enumerated in the same order as the comments of the previous investigations listed above.

1. The bleedings to which our dogs have been subjected were so regulated that whenever the concentration of serum protein rose to 4.2 per cent or above, a *calculated* volume of blood was removed to reduce the level to 3.5 per cent. This concentration of the blood protein has been arbitrarily chosen and will hereafter be called the basal level. Daily determinations of the serum protein concentration in duplicate by a nephelometric technique and weekly determinations of the blood volume by a dye method were carried out *previous* to the bleedings. With the bleeding apparatus finally devised for the study, the volumes of blood removed averaged easily within ± 1 per cent of the calculated desired amounts. The description of the apparatus and discussion of the validity of the methods are presented in detail elsewhere (12). With a favorable diet the average serum protein concentration *previous* to the bleedings was found to be distinctly higher than that recorded subsequent to the feeding with a less effective ration; the point in our technique to be emphasized, however, is that the actual bleedings were such as to *reduce* the serum protein concentration as far as possible to a constant level. Thus, by conducting the bleedings *quantitatively* in our work, the intensity of the

TABLE I

Quantitative Plasmapheresis in the Study of the Influence of Diet upon the Regeneration of Serum Protein: Representative Periods

Period	Date	Blood volume	Hematocrit	Serum protein concentration	Bleeding	Cell exchange		Citrate plasma		Serum protein removal per week (concentration)*
						bled	infected	citrate	plasma	
	1936	cc.	per cent	per cent	cc.	cc.	cc.	cc.	cc.	gm.
XII Week of initial depletion (dog 3)	Oct. 21	1315†	48.9	6.20	329	161	140	37	168	43.5 (4.44%)
	22	—	47.1	5.89	329	155	156	37	174	
	23	—	40.6	4.65	329	134	150	37	195	
	24	—	42.6	4.49	324	138	129	37	186	
	25	—	38.7	4.22	329	127	133	37	202	
	26	1212‡	35.6	3.73	81§	28	123	9	53	
	27	—	—	3.43	—	—	—	—	—	
XVII Equilibrium week with basal diet (dog 3)	Dec. 9	1128†	39.3	4.21	192	76	185	21	116	15.7 (4.20%)
	10	—	—	3.58	—	—	—	—	—	
	11	—	—	4.06	—	—	—	—	—	
	12	—	43.1	4.16	183	79	74	20	104	
	13	—	—	3.84	—	—	—	—	—	
	14	1143‡	43.0	4.56	266	112	77	29	154	
	15	—	—	3.77	—	—	—	—	—	
X Equilibrium week with protein diet (dog 4)	June 17	1372†	37.9	5.33	472	179	246	53	293	68.5 (4.46%)
	18	—	38.0	4.91	393	149	229	44	244	
	19	—	40.5	4.94	400	162	147	45	238	
	20	—	38.1	5.14	436	167	157	49	269	
	21	—	33.4	4.79	370	128	176	41	242	
	22	1275	35.1	4.85	383	134	228	43	249	
	23	—	—	4.65	—	—	—	—	—	

* Corrected for the citrate and calcium chloride dilutions.

† This value obtained 2 days previously was used in determining the size of each of the hemorrhages performed during the period.

‡ This blood volume was employed in calculating the amount of blood to be withdrawn during each plasmapheresis conducted in the subsequent period.

§ This bleeding was calculated to reduce the serum protein concentration to the basal level of 3.5 per cent; the other hemorrhages carried out during this period were one-fourth of the blood volume (1315 cc.).

stimulus for serum protein regeneration has been maintained as constant as possible.

Subsequent to each plasmapheresis an aliquot of the citrated plasma is removed and pooled with the others obtained during that experimental period. For the conversion of the plasma to serum a 6.4 per cent $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$ solution is added, 1 cc. per each cc. of the citrate solution present, resulting in the complete precipitation of the insoluble fibrin (13). The concentration of the protein in the expressed serum is determined by the direct gravimetric technique of Bierry and Vivarro (14), modified by Guillaumin, Wahl and Laurencin (15). This is the most accurate method known for the determination of the serum protein concentration.

To illustrate our quantitative approach to the problem several representative periods are given in Table I. Period XII, obtained with dog 3, is characteristic of the initial weeks during which the serum protein concentrations of the dogs are reduced to the basal level and the reserve serum protein stores depleted. The protein-free diet is administered during this period at a high level of caloric intake. Period XVII, obtained with the same animal, is representative of the basal equilibrium weeks during which the animals are fed the protein-free diet and subjected to quantitative plasmapheresis; each bleeding is calculated to reduce the serum protein concentration to the basal level of 3.5 per cent. A typical equilibrium period during which the animal is fed a favorable protein diet is illustrated in period X obtained with dog 4. Plasmapheresis was conducted quantitatively as in the basal equilibrium period. The animal was fed daily the serum protein increment diet (16) at the maintenance level of 70 calories per kilo of body weight.

2. Our "synthetic" artificial basal diet is essentially free of protein. Its composition is given in Table II. With respect to the vitamin adjuvants, it was desirable that even these should not introduce any appreciable amount of protein. The tablets furnishing the fat soluble vitamins were devoid of protein nitrogen. The dosage of the vitamin B (B_1) preparation proved to be adequate and, although it contained 0.94 per cent nitrogen, it introduced no appreciable source of dietary protein. The sample of liver extract 343, employed in the present study as a source of vitamin G (B_2), contained 8.1 per cent of nitrogen of which 45 per cent is potentially protein nitrogen (17).

TABLE II
Protein-Free Diet
Basal Dietary Mixture

Foodstuff	Amount	Calories	
		Absolute	Per cent
	<i>per cent</i>		
Sucrose.....	55.10	220	44.7
Dextrin.....	18.17	73	14.7
Lard.....	16.65	150	30.4
Butter*.....	6.54	50	10.2
Bone ash.....	2.36	—	—
Salt mixture (25).....	1.18	—	—
Total.....	100.00	493	100.0

1 gm. of this dietary mixture furnishes 4.93 calories.

* The value of 7.7 calories per gm. was used in calculating the amount of calories furnished by the butter (18).

Vitamin Supplements

For vitamins A and D two tablets of cod liver oil concentrate² were given daily per 5 to 7 kilo body weight of dog.

As a source of vitamin B (B₁) an extract of rice polishing³ was administered. The sample contained 56 international units per gm and was given daily to the extent of 56 mg. per kilo of body weight.

The vitamin G (B₂) requirement of the dog was satisfied by liver extract 343.⁴ This preparation was given daily in doses of 210 mg. per kilo of body weight.

At all the feedings the vitamin adjuvants⁵ were administered apart from the food mixture, thus insuring an adequate vitamin intake to the animals. These supplements furnish an additional 1.5 calories per kilo of body weight daily.

² Kindly furnished by the Health Products Corp., Newark. During the administration of the protein-free diet containing butter, it is not essential to feed these tablets for the purpose of supplying an adequate intake of fat soluble vitamins. However, it was desirable to keep the materials containing the vitamin supplements constant throughout all experimental periods.

³ Ryzamin, obtained from the Burroughs Wellcome Co., Tuckahoe, New York.

⁴ Kindly furnished in the form of a dry powder by Eli Lilly and Co., Indianapolis.

⁵ It was found convenient to prepare an aqueous suspension of 75 gm. of liver extract 343 and 20 gm. of ryzamin in 250 cc. water. This suspension, adjusted to a pH just acid to litmus, was given orally in the dosage of 0.7 cc. per kilo of body weight.

Thus, this preparation yielded a slight but appreciable amount of "protein," estimated to be about 50 mg. per kilo of body weight.

With a diet such as that in Table II, it has been possible repeatedly, in four dogs, to deplete the organism of its reserve serum protein stores within the 1st week of plasmapheresis, bleeding daily one-fourth of the animal's blood volume. It was noted that during the periods when the above diet was fed *ad libitum* the animals ate considerably more than their caloric requirements under normal conditions, the voluntary intake of calories averaging approximately 90 calories per kilo of body weight. This, of course, exerted a protein-sparing action in the body and tended to reduce to a minimum the stimulus for regeneration of serum protein. Thus, the number and size of bleedings per week of the animals partaking of this ration were also minimal. These qualifications are to be desired in the basal diet.

3. "Synthetic" artificial diets, of which the chemical nature and exact proportions of all nutrients were known, have been used in this study. Only one constituent in the diet was varied at a time, namely, the protein to be evaluated.

4. Furthermore, in all cases the proteins under investigation were analyzed, the appropriate factor (19) employed for each, and then incorporated into the diet. Thus, errors resulting from use of the conventional calculation, $6.25 \times$ percentage of nitrogen, were avoided. The question arose at what level the proteins of interest should be fed. It was finally decided in all cases to feed an *equal* absolute amount of the respective proteins above the minimum of each required to establish nitrogen equilibrium.

5. The dogs were fed daily 70 calories in the form of the experimental diets per kilo of body weight so that the *dietary* stimulus for the regeneration of serum protein was constant from day to day. These diets were constructed on the kilo-unit plan, as first described by Cowgill (20) and designed to yield 80 calories per kilo. However, inasmuch as our dogs were kept in metabolism cages, and their activity thus reduced, 70 calories per kilo of body weight were found to be optimum for maintenance. The weight of the animal employed in calculating the amount of food to be administered was that essential for an optimal nutritive condition, as estimated by the nutritive index formula also described by Cowgill (21). The vitamin adjuvants yielded in addition 1.5 calories daily per kilo of body weight.

6. Having previously determined for each protein evaluated the minimum required for nitrogen equilibrium (22), it has been possible in this study to correct for that fraction of dietary protein essential for the general nitrogen requirements of the organism. The increment of protein tested, above the amount required for nitrogen equilibrium, is then theoretically available for the regeneration of the needed serum protein. It has been found that feeding the protein at that level, which is the *minimum* for nitrogen equilibrium, *or less*, results in the regeneration of serum protein, but this is now at the expense of that portion essential for the normal maintenance metabolism of the organism. The ratio of (a) the amount of serum protein per week removed by the bleedings, above that regenerated by the animal when eating the basal protein-free diet, to (b) the dietary protein increment, *i.e.*, the amount above that required for nitrogen equilibrium, is termed in this study the potency ratio. It will be observed that when using this particular ratio, the protein most potent has the highest potency ratio. This point is mentioned here because the ratio formulated by Whipple and associates bears an inverse relationship. In all cases where a new diet was administered, the 1st week was regarded merely as the adjustment period. The subsequent week, considered as the period of constant and maximal response to the dietary stimulus, furnished the data used in the calculation of the potency ratio.

7. In the present studies confirmatory evidence has been sought for results obtained following each dietary change. Because the experiments extended over a long time interval, and the technical difficulties were so great and exacting, it has proved impossible within the time requirements and the available facilities to carry out these investigations satisfactorily on more than two or three dogs. Whipple and coworkers likewise emphasize this fact.

RESULTS

Standardization of the Dogs: Reserve Serum Protein Stores

Two dogs were used in this study. At a nutritive index of 0.30 (21), dog 3, a female hound, weighed 16.70 kilos, and dog 4, a female collie, 17.30 kilos. The first objective was to deplete the animals of their reserve serum protein stores. While eating the basal protein-free diet *ad libitum* (see Table II), the dogs were subjected to plasmapheresis.

During the 1st week (period I) of plasmapheresis, one-fourth of the animal's

TABLE III

Standardization of the Dogs: the Depletion of the Reserve Serum Protein Stores

Dog No.....	3					4		
Weight with nutritive index 0.30...	16.70 kg.					17.30 kg.		
Diet	Protein-free					Protein-free		
Period	I Depletion	II Equilibrium	III Equilibrium	X Depletion	XI Equilibrium	I Depletion	II Equilibrium	III Equilibrium
Average daily intake of diet* per kg., calories.....	88	107	87	89	73	124	102	84
Average weight of dog, kg....	16.85	16.73	16.82	17.47	17.36	17.71	17.44	17.43
Blood volume, cc.....	1335	1170	1145	1360	1227	1605	1605†	1260
Plasma volume, cc.....	651	650	664	835	755	828	828†	768
Average hematocrit, per cent.....	50.1	45.1	42.8	41.0	40.9	46.5	46.6	37.1
Average serum protein concentration, ‡ per cent.	5.45§	4.12	4.16	4.62	4.03	5.11**	3.94	3.94
Total bleeding, cc.....	1962	489	655	1856	589	2260	538	524
Cell exchange Bled, cc.....	983	221	285	767	241	1041	250	191
Injected, cc....	1017	330	323	853	293	1036	371	336
Citrated plasma Citrate, cc....	216	54	76	207	65	263	59	58
Plasma, cc....	980	268	370	1089	348	1217	288	334
Serum protein concentration, †† per cent....	5.02	4.16	4.17	4.33	3.94	4.64	3.82	3.85
Serum protein removed per week, gm....	49.2	11.2	15.4	47.2	13.7	56.4	11.0	12.8

blood volume was removed daily. In the subsequent weeks (periods II and III) bleedings were performed only when the serum protein levels rose to or above 4.2 per cent. The bleedings were calculated to reduce the concentrations to 3.5 per cent. An excess of 100 cc. of modified Locke's solution (3) was injected at the conclusion of each of the red cell infusions. Occasionally, donors' cells were injected to maintain a normal hematocrit. The details of the plasmapheresis and the nutritive histories of the dogs are summarized in Table III.

In both dogs the total amounts of serum protein removed per week during the 2nd and 3rd weeks were approximately the same. This indicates that dogs fed the high caloric, protein-free diet and subjected to plasmapheresis, by which one-fourth of the blood volume is removed daily, can be depleted of the reserve serum

* The vitamin adjuvants furnished in addition 1.5 calories per kilo of body weight.

† The blood volume for this period was not determined; the value obtained during the previous week was employed in calculating the size of the bleedings conducted during period II.

‡ These values were obtained by the nephelometric method as prerequisites for determining daily the size of the hemorrhages (12). The serum protein concentrations were averaged regardless of whether or not a plasmapheresis was conducted that day.

§ Reduced from 6.82 to 4.04 per cent.

|| Reduced from 6.21 to 3.89 per cent.

** Reduced from 6.28 to 3.88 per cent.

†† In all cases the serum protein concentrations in the pooled plasma aliquots, after corrections had been made for the presence of the citrate and calcium chloride solutions, were approximately 10 per cent less than the weighted average results obtained daily previous to the hemorrhages. The possibility of an immediate dilution of the blood due to the entrance of tissue fluid, occurring during the actual bleeding, was investigated. Initial blood samples were taken a few minutes before the bleeding; the final samples were the last 2 cc. of blood drawn from the animal. Using the change in the serum protein concentration as the criterion, a valid procedure because the storage factor in these dogs was eliminated, it appeared that a dilution was taking place. The average decrease in the serum protein concentration in the course of four such studies was 8 per cent, the bleedings averaging approximately one-fourth of the blood volumes. However, this physiological response to hemorrhage was not adequate for explaining the discrepancies noted. Especially during periods of rapid regeneration of serum protein the initial values of the protein concentration tended to be actually slightly higher than the results obtained by the usual determinations conducted earlier in the day. It was also demonstrated that the citrate solution, used as the anticoagulant, did not cause any appreciable dehydration of the red cells to produce a plasma dilution.

protein stores within 1 week. To attain the basal level, dog 3 had to be bled that quantity of blood containing 49.2 gm. of serum protein, and dog 4, 56.4 gm. of serum protein. Once the stores are depleted (after the 1st week by this technique), it is found that the dog fed the protein-free diet will regenerate a minimal and constant amount of serum protein each week while the serum protein concentration is maintained at the basal level. The fact that the amount of serum protein removed in the bleedings of the 3rd week is approximately equal and certainly not less than that recorded for the 2nd week indicates that the dog has been depleted of its serum protein reserve stores within the 1st week, and the basal outputs thereafter are equilibrium values. Thus, it is noted that dog 3 was bled that amount of blood containing 13.9 gm. of serum protein (average value), and dog 4, 11.9 gm., for the maintenance of a constant basal level.

TABLE IV

Scrum Protein Regeneration in Dogs Subsisting on the Protein-Free Diet and Subjected to Quantitative Plasmapheresis: Equilibrium Values

Dog No.	Period	Basal output per week	Average per week
		<i>gm.</i>	<i>gm.</i>
3	II	11.2	13.4
	III	15.4	
	XI	13.7	
	XIII	11.2	
	XVII	15.7	
4	II	11.0	9.6
	III	12.8	
	XII	4.4	
	XVI	10.0	

The reproducibility of the basal output of serum protein by dog 3 was investigated 10 weeks later. The animal had just completed 2 weeks on the serum protein increment diet (16) and 1 week on our stock casein diet (21). The results are also given in Table III, periods X and XI. The value, 13.7 gm., agrees very well with the others recorded previously.

Rather than maintain the dogs on a long régime of repeated plasmapheresis in the subsequent studies, it was deemed advisable to give them a rest period after each evaluation so that the serum protein concentrations might return to normal again. During the first 2 weeks of these biological assays the protein-free diet was administered at a high caloric level. The 1st week was devoted to the depletion of the reserve serum protein stores; the subsequent week was considered to be a basal equilibrium period. Table IV gives the values for these comparable periods and the average. The relative constancy of these data seems to emphasize the validity of the method of experimentation.

In the evaluations of any dietary factor, it therefore becomes essential that these values, 13.4 and 9.6 gm. for dogs 3 and 4, respectively, be subtracted from the total grams of serum protein removed per week to arrive at the fractions arising solely from the dietary stimulus. Such a procedure appears to be logical because the basal diet is free of protein and, being fed at a high level of caloric intake, its use reduces protein catabolism to a minimum.

Reserve Serum Protein Stores.—Because our findings with respect to the reserve serum protein stores do not agree with those reported by other investigators, it appears advisable to present in detail a representative calculation based on our data.

Dog 4 initially had a plasma volume of 828 cc. and a serum protein concentration of 6.28 per cent.

Then

$$828 \times 0.0628 = 52.1 \text{ gm. of circulating serum protein.}$$

The serum protein level of this animal was reduced from 6.28 to approximately 3.90 per cent.

Then

$$6.28 - 3.90 = 2.38 \text{ per cent, and}$$

$$\frac{2.38}{6.28} \times 52.1 = 19.8 \text{ gm. of circulating serum protein removed in the bleedings.}$$

This dog was fed the protein-free diet during the initial depletion period and subsequent equilibrium weeks. With such a ration the animal regenerated an average of 9.6 gm. of serum protein per week.

Therefore,

$$52.1 - 19.8 - 9.6 = 22.7 \text{ gm. of reserve serum protein, stored as such or potentially so, in dog 4.}$$

Thus, this animal possessed a reserve store of serum protein material equivalent to about 43 per cent of the total amount of circulating serum protein normally present.

Dog 3 had initially 44.4 gm. of circulating serum protein, of which 18 gm. were removed in the bleedings in order to reduce the serum protein concentration to the basal level. When fed the protein-free diet and subjected to plasmapheresis, this animal regenerated an average of 13.4 gm. of serum protein per week. Therefore, 13.0 gm. of serum protein constituted this dog's reserve store. This is equivalent to approximately 29 per cent of the total serum protein normally present in the plasma.

In a study being conducted at present on the mechanism of serum protein regeneration with another animal, dog 4-4, a fox-terrier weighing 10.0 kilos, a similar procedure was followed in calculating this animal's reserve serum protein store. The amount of circulating serum protein was found to be 37.2 gm. In

order to reduce the concentration of the blood protein to the basal level 15.5 gm. were removed in the bleedings. With the protein-free diet this dog could regenerate 9.2 gm. of serum protein per week. Therefore, 12.5 gm. of serum protein were present as such, or potentially so, in the form of reserve protein. This value, approximately 34 per cent of the total circulating serum protein, agrees very well with those recorded for dogs 3 and 4.

It is recognized that these calculations are not above criticism. The most serious objection is the assignment of a value for the basal output of serum protein to the 1st week of the bleedings. This basal output is a constant value only when the basal level of the serum protein concentration is constant. When this is relatively high (above 5 per cent) and when the dog is fed the protein-free diet, the stimulus for the production of new serum protein is negligible (16), so that the subtraction of the basal output from the total removed must result in a significant, negative error. However, to compensate for this noneffectiveness of the protein-free diet during the early part of this 1st week, there is a carry over of the favorable effects of the stock diet previously fed to the dogs. This factor, therefore, produces a significant and positive error which tends to neutralize the negative error discussed. The calculated value, approximately 30 to 40 per cent of the circulating protein, for the amount of reserve store of serum protein building material is probably accurate to within ± 10 per cent of the normal total circulating serum protein. This is in marked contrast to the statement by Whipple and associates (5) that the dog has on the average a reserve store of serum protein building material two or three times as much as is normally present in the circulation.

Time Required to Secure Equilibrium Values

Whipple and associates (4-6) have reported that a favorable dietary factor, when administered to a dog subjected to plasmapheresis, causes an intense stimulus for the regeneration of serum protein. The maximal response to this stimulus becomes manifest only after 3 to 7 days of feeding. It was our purpose, first of all, to determine the amount of regeneration of serum protein during several consecutive weeks under our conditions of repeated, quantitative plasmapheresis and constant intake of experimental diet, in order to ascertain which week should furnish the data to be used in calculating the potency ratio.

The initial investigation was made with beef serum protein fed in an amount proved by previous investigation with normal dogs to be just adequate for the attainment of nitrogen equilibrium (22). The diet contained the protein in quantity sufficient to furnish 8.5 per cent of the caloric intake. This value was demonstrated experimentally to be adequate for dog 4; dog 3, however, required 9.1 per cent of the caloric intake in the form of serum protein. The composition of the serum protein diet fed to the dogs is given in Table V.

Subsequent to period III (*i.e.*, the 3rd week on the protein-free diet with plasmapheresis performed quantitatively), dog 3 was fed the serum protein diet at a level similar to that employed with the metabolism dogs, namely, 70 calories

TABLE V
Serum Protein Diet: Kilo-Unit

Foodstuff	Amount	Calories		Composition
		Absolute	Per cent	
	<i>gm.</i>			<i>per cent</i>
Serum protein*	2.22 (1.70)	6.8	8.5	13.45 (10.3)
Crisco	3.79	34.1	42.6	23.00
Sucrose	5.02	20.1	25.1	30.45
Dextrin	4.76	19.0	23.8	28.86
Bone ash	0.40	—	—	2.42
Salt mixture (25)	0.30	—	—	1.82
Total	16.49	80.0	100.0	100.00

The numbers in parentheses are the absolute values.

1 gm. of this diet furnishes 4.85 calories.

The vitamin adjuvants are the same as those supplementing the basal dietary mixture (see Table II).

* Analysis of the protein indicated 12.2 per cent nitrogen, or 76.4 per cent purity. The protein was isolated from beef serum (22).

per kilo of body weight. Plasmapheresis was continued according to the quantitative technique described (12). The animal was subjected to this procedure for 3 weeks. Table VI summarizes the experimental findings.

The delay, reported by Whipple and collaborators (5), following the administration of a potent dietary factor was also observed in this study. The fact that the amount of serum protein removed in the bleedings of the 3rd week (36.8 gm.) was not greater than that obtained in the 2nd week (40.8 gm.), indicates that the feeding of the dietary component for 2 weeks under these experimental conditions is sufficient

for its proper evaluation. The 2nd week is one of constant and maximal response. In the succeeding biological assays therefore, the 1st week has always been regarded as the adjustment period and the 2nd week furnishes the data used in the calculation of the potency ratio.

Dog 4 was subjected to a similar procedure. During the 1st week (period IV) on the serum protein diet, it was necessary to remove 27.0 gm. of serum protein in the bleedings in order to maintain a constant basal level. It was at this stage of the investigation that incompatibilities were first noted between the sera of the recipients and the cells of the donors (23). With dog 3 it was possible to complete period VI satisfactorily. In the case of dog 4, the equilibrium week, period V, was not finished. Repeated shock reactions in both recipients subsequent to the red cell infusions from the donor animals necessitated a discontinuance of the study until satisfactory donors were obtained. During this interval of 3 weeks both dogs subsisted on the basal protein-free diet *ad libitum* in order to prevent the accumulation of reserve stores of serum protein building material.

When the study was resumed with dog 4, the protein-free diet was administered for an additional week (period VI) during which time quantitative plasmapheresis was performed to reduce the serum protein concentration to the basal level and to deplete the body of its reserve serum protein stores. Only two bleedings, resulting in the removal of 11.0 gm. of serum protein, were essential for accomplishing this. When the serum protein diet was fed again, it was noted after the adjustment period VII that 48.6 gm. of serum protein had to be removed in order to maintain the basal level constant. Because our technique involves quantitative plasmapheresis and constant dietary intake, the daily value of the serum protein concentration is an accurate index of the response of the animal to the experimental régime. A consideration of such data in the protocols for periods VII and VIII (22) indicated that equilibrium values were attained 5 days after the administration of the protein diet. It was therefore considered not essential to extend the study through another experimental period.

In Table VI are summarized the details of plasmapheresis and the nutritive histories of the dogs, while subsisting on the serum protein diet. The food was offered to the dogs about 3 hours after plasmapheresis because feeding the animal shortly before bleeding (within 4 to 5 hours) usually resulted in vomiting during or immediately after the plasmapheresis. In the case of the protein-free diet, fed *ad libitum*, the food pans were always removed from the cage 4 to 5 hours before the bleedings.

DISCUSSION AND CONCLUSIONS

From the studies reported it appears that the minimal time requirement for the biological assay of a protein in promoting serum protein regeneration is 4 weeks. By the administration of an artificial protein-

free diet with concomitant plasmapheresis, during which the animals are bled one-fourth of their blood volumes daily, it has been possible

TABLE VI
Time Required for Dogs Fed a Favorable Diet and Subjected to Quantitative Plasmapheresis to Attain Equilibrium Values

Dog No.....	3			4	
Weight with nutritive index 0.30.....	16.70 kg.			17.30 kg.	
Diet.....	Serum protein			Serum protein	
Period.....	IV Adjust- ment	V Equili- brium	VI Equili- brium	VII Adjust- ment	VIII Equili- brium
Daily intake of diet,* per kg., calories....	70	70	70	70	70
Average weight of dog, kg.....	16.98	17.10	17.23	17.27	17.18
Blood volume, cc.....	1145	1158	1140	1335	1425
Plasma volume, cc.....	664	695	735	683	880
Average hematocrit, per cent.....	40.8	40.4	36.9	43.8	42.6
Average serum protein concentration,* per cent.....	4.24	4.53	4.37	4.41	4.66
Total bleeding, cc.....	939	1692	1474	1281	2062
Cell exchange					
Bled, cc.....	387	687	546	558	880
Injected, cc.....	648	755	642	510	1087
Citrated plasma					
Citrate, cc.....	102	187	163	143	230
Plasma, cc.....	552	1005	928	723	1182
Serum protein concentration,* per cent.....	4.00	4.06	3.96	4.03	4.11
Serum protein removed per week, gm...	22.1	40.8	36.8	29.2	48.6

* The same qualifying statements, added as footnotes to Table III, apply also to this table.

repeatedly to reduce the serum protein concentration to the basal level and to deplete the organism of its reserve serum protein stores

for its proper evaluation. The 2nd week is one of constant and maximal response. In the succeeding biological assays therefore, the 1st week has always been regarded as the adjustment period and the 2nd week furnishes the data used in the calculation of the potency ratio.

Dog 4 was subjected to a similar procedure. During the 1st week (period IV) on the serum protein diet, it was necessary to remove 27.0 gm. of serum protein in the bleedings in order to maintain a constant basal level. It was at this stage of the investigation that incompatibilities were first noted between the sera of the recipients and the cells of the donors (23). With dog 3 it was possible to complete period VI satisfactorily. In the case of dog 4, the equilibrium week, period V, was not finished. Repeated shock reactions in both recipients subsequent to the red cell infusions from the donor animals necessitated a discontinuance of the study until satisfactory donors were obtained. During this interval of 3 weeks both dogs subsisted on the basal protein-free diet *ad libitum* in order to prevent the accumulation of reserve stores of serum protein building material.

When the study was resumed with dog 4, the protein-free diet was administered for an additional week (period VI) during which time quantitative plasmapheresis was performed to reduce the serum protein concentration to the basal level and to deplete the body of its reserve serum protein stores. Only two bleedings, resulting in the removal of 11.0 gm. of serum protein, were essential for accomplishing this. When the serum protein diet was fed again, it was noted after the adjustment period VII that 48.6 gm. of serum protein had to be removed in order to maintain the basal level constant. Because our technique involves quantitative plasmapheresis and constant dietary intake, the daily value of the serum protein concentration is an accurate index of the response of the animal to the experimental régime. A consideration of such data in the protocols for periods VII and VIII (22) indicated that equilibrium values were attained 5 days after the administration of the protein diet. It was therefore considered not essential to extend the study through another experimental period.

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Diet.....	Serum protein			Serum protein	
Period.....	IV Adjust- ment	V Equili- brium	VI Equili- brium	VII Adjust- ment	VIII Equili- brium
Daily intake of diet,* per kg., calories....	70	70	70	70	70
Average weight of dog, kg.....	16.98	17.10	17.23	17.27	17.18
Blood volume, cc.....	1145	1158	1140	1335	1425
Plasma volume, cc.....	664	695	735	683	880
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* The same qualifying statements, added as footnotes to Table III, apply also to this table.

repeatedly to reduce the serum protein concentration to the basal level and to deplete the organism of its reserve serum protein stores

within 1 week. The subsequent week is an equilibrium period, indicative of the amount of serum protein that can be regenerated with no protein in the diet and with protein catabolism reduced to a minimum by the consumption of the protein-free diet at a high level of caloric intake. The last 2 weeks of the assay are devoted to the administration of the protein diet; the 1st week on this dietary régime is regarded merely as the adjustment period; the subsequent week, being the period of constant and maximal response to the dietary stimulus, furnishes the data to be used in the calculation of the potency ratio. During the last 3 weeks of the assay plasmapheresis is performed quantitatively so that the stimulus for the regeneration of serum protein *in vivo* may be constant from day to day.

It is highly desirable to give the animal a rest period after each biological assay so that a normal serum protein concentration may be attained. In another investigation (22) we have some evidence indicating that the animal subjected to long continued, repeated plasmapheresis will fail to regenerate serum protein as efficiently as during the initial tests.

In our studies the animals were initially in an optimal nutritive state. Using the weight value as criterion, it is evident that dogs subjected to our feeding technique have no difficulty maintaining a healthy nutritional status.

In the present investigation, as well as those conducted by the pioneer investigators in this field (4-6), it has been found necessary to inject periodically an excess of red blood cells in order to prevent the animal from becoming anemic. The hemoglobin thrown into the circulation by the hemolysis of injected erythrocytes may undergo hydrolysis and the globin fraction therefore be rendered theoretically available as a source of amino acids for promoting the regeneration of serum protein. It is our belief that this "globin variable" exhibits not only an additive but also a supplementary effect upon the dietary protein, so that the result obtained cannot be referred solely to the dietary factor. In a recent paper (24), Whipple and his associates state that hemoglobin, when given intravenously, is simply "broken down and thrown away as extra nitrogen in the urine." If their experimental results could be so interpreted, then the globin variable would be of no significance. However, a critical examination of this

study (22), led us to conclude that the data were insufficient to warrant such an interpretation.

SUMMARY

1. From a consideration of previous work related to the problem of the influence of diet upon the regeneration of serum protein, a plan of study is described which eliminates the many variables shown to be operating in the studies conducted by these pioneer workers.

2. By the administration of a protein-free diet at a high level of caloric intake to the dog subjected to plasmapheresis during which one-fourth of the blood volume of the animal is withdrawn daily, it is possible to reduce the serum protein concentration to the basal level (3.5 to 4.2 per cent) and to deplete the organism of its reserve stores of this protein within 1 week. The subsequent week has been demonstrated to be an equilibrium period.

3. The dog contains a reserve store of serum protein building material equivalent to about 30 to 40 per cent of the total amount normally present in the circulation.

4. When fed the protein-free diet and when subjected to quantitative plasmapheresis, whereby the basal level of the serum protein concentration is maintained constant, the dog is able in 1 week to regenerate approximately 20 to 30 per cent of the total amount of this blood protein normally present in the plasma.

5. The administration of a diet favorable for promoting the regeneration of serum protein requires approximately 4 to 5 days before a constant and maximal response to the dietary stimulus is obtained. Equilibrium data are yielded during the 2nd week, and these are employed in calculating the potency ratio of the dietary protein.

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THE INFLUENCE OF DIET UPON THE REGENERATION OF SERUM PROTEIN

II. THE POTENCY RATIOS OF SERUM PROTEIN, LACTALBUMIN AND CASEIN, AND THE EFFECT OF TISSUE PROTEIN CATABOLISM ON THE FORMATION OF SERUM PROTEIN

BY DANIEL MELNICK,* PH.D., GEORGE R. COWGILL, PH.D., AND
ETHEL BURACK,** PH.D.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven)

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In the evaluation of dietary proteins for their influence upon the regeneration of serum protein, it should be taken into consideration that the protein which is ingested must serve in two ways, namely, (a) in meeting the minimal requirement for nitrogen equilibrium of the entire organism and (b) in the formation of new serum protein. Inasmuch as proteins differ with respect to the amounts required for (a), it is obvious that preliminary determinations of the quantities needed for this purpose should be conducted before tests for function (b) are made. If (a) is known, the evaluation with respect to (b) may be carried out by feeding the same absolute amounts of the test proteins as increments above the quantities required for (a). Theoretically the relative potencies of the various proteins for the regeneration of serum protein should then be evidenced solely by the effects obtained when the test increment quantities are fed. Thus, the potency ratio in this study has been expressed as the ratio of the amount of serum protein per week removed by the bleedings above that regenerated by the animal when eating the basal protein-free diet, to the dietary protein increment, *i.e.*, the amount above that required for nitrogen equilibrium.

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** Standard Brands Inc. Fellow, 1934-1936.

The determinations on normal dogs of the protein minima for nitrogen equilibrium with serum protein, casein, lactalbumin and gliadin are described elsewhere (1). The original purpose was to relate the average values yielded by these experiments to the data obtained with the dogs subjected to plasmapheresis. Only after the preliminary nitrogen balance studies were completed was it learned that the protein minima are not exactly the same for different animals. Therefore, it was deemed necessary to determine the minimum for each dog subjected to the plasmapheresis régime. The minimal values as determined on dogs 3 and 4 are given in Table I.

TABLE I
Protein Minima for Nitrogen Equilibrium

Dog No.	Protein	Calories from protein
		<i>per cent</i>
3	Serum protein	9.1
	Lactalbumin	7.4
	Casein	9.1
4	Serum protein	8.5
	Lactalbumin	6.65
	Casein	8.9

Previously plasmapheresis was performed on these dogs fed in both cases a serum protein diet in which the protein furnished 12.9 per cent of the total caloric intake. Fortunately, this circumstance did not invalidate the final calculations of the potency ratio of serum protein because the "increment diet"¹ contained far more than the minimum for each of the dogs, and appropriate calculations could be made to determine the actual increments available for serum protein regeneration. Thus, dog 3 received in the diet a protein increment amounting to 0.75 gm. per kilo-unit; and dog 4, 0.87 gm. per kilo-unit. Inasmuch as each dog is its own control, the other diets were constructed in such a manner that their protein increments, above that required for nitrogen equilibrium, were *equal* for the different proteins. Therefore, in the evaluation of serum protein, lactalbumin and casein, dog 3 received per week in every case an excess of 76 gm. of the protein above the nitrogen minimum level. Dog 4 received 92 gm. It would have been

¹ To differentiate the protein diets administered during the plasmapheresis experiments from those fed in the nitrogen balance studies, the expression increment diets is used. In the subsequent discussion the expression is employed without quotation marks.

desirable to have fed both animals the same absolute increments of the proteins tested. However, the absolute amounts administered are comparable and confirmatory studies are significant. The protein increments replaced isodynamically an equivalent part of sucrose in the diet so that, calorically speaking, the dogs ate the same amount of food. In all cases the protein diets were fed daily at the maintenance level of 70 calories per kilo of body weight. The weight of the animal, used in estimating the amount of food to be fed, was that calculated for the animal when in an optimal nutritive condition with a nutritive index value equal to 0.30 (2). The vitamin adjuvants were not varied. Thus the only variable in the diet, so far as known, was this protein increment. Inasmuch as the nature of the protein in the increment and in the basal minimum diet was the same, no supplementary action to offset any amino acid deficiencies could take place. These diets, constructed according to the kilo-unit plan (3), are given in Table II. The values in the parentheses indicate the absolute amounts of protein based on actual analyses for nitrogen, and were the amounts used in estimating calories.

The serum protein was isolated from beef serum (1); the casein² and lactalbumin³ were commercial preparations. The vitamin adjuvants and their mode of administration are described elsewhere (4).

The smallest amount of gliadin required for nitrogen equilibrium was determined as 21.1 per cent of the caloric intake. Gliadin, deficient in the essential amino acid lysine, would have been a splendid protein to employ in the study of serum protein regeneration. Would it promote any regeneration above that observed with the protein-free diet? If it did, would the amino acid composition of the regenerated serum protein differ from the normal? Such an experiment would offer another means for testing Block's *Anlage* theory (5), "the primary importance of the basic amino acids (as evidenced by the constancy of their ratios) in the genetic and embryological development of the tissue protein as it exists in protoplasm."⁴

However, due to the high level of gliadin required simply for the attainment of nitrogen equilibrium and due to the fact that unexpected toxic reactions were manifested during the feeding of high gliadin diets (1), no attempt was made to assay this protein for its value in promoting serum protein regeneration. Furthermore, the globin variable—the slow but continuous break-down of the injected erythro-

² Obtained from the Lister Bros., New York.

³ Obtained from the National Milk Sugar Co., Bainbridge, New York.

⁴ The material in parentheses has been inserted by us.

TABLE II
Protein Diets Used in the Present Study Based on the Kilo-Unit Plan*

Increment diet (Recipient)	Foodstuff	Amount	Calories		Composition	Calories per gram of diet
			Abs- olute	Per cent		
		gm.			per cent	
Serum protein (dog 3 and dog 4)	Serum protein (76.4%; 12.20% N)	3.36 (2.57)	10.3	12.9	20.00 (15.30)	4.77
	Lard	3.79	34.1	42.6	22.62	
	Sucrose	8.90	35.6	44.5	53.20	
	Bone ash	0.40	—	—	2.39	
	Salt mixture (22)	0.30	—	—	1.79	
Total.....		16.75	80.0	100.0	100.00	
Lactalbumin (dog 3)	Lactalbumin (76.0%; 11.68% N)	2.94 (2.23)	8.9	11.1	17.60 (13.40)	4.80
	Lard	3.77	34.0	42.5	22.60	
	Sucrose	9.27	37.1	46.4	55.60	
	Bone ash	0.40	—	—	2.40	
	Salt mixture	0.30	—	—	1.80	
Total.....		16.68	80.0	100.0	100.00	
Lactalbumin (dog 4)	Lactalbumin (76.0%; 11.68% N)	2.89 (2.20)	8.8	11.0	17.36 (13.20)	4.81
	Lard	3.77	34.0	42.5	22.64	
	Sucrose	9.30	37.2	46.5	55.80	
	Bone ash	0.40	—	—	2.40	
	Salt mixture	0.30	—	—	1.80	
Total.....		16.66	80.0	100.0	100.00	
Casein (dog 3)	Casein (84.1%; 13.37% N)	3.05 (2.57)	10.3	12.9	18.55 (15.60)	4.87
	Lard	3.77	34.0	42.5	22.90	
	Sucrose	8.93	35.7	44.6	54.30	
	Bone ash	0.40	—	—	2.43	
	Salt mixture	0.30	—	—	1.82	
Total.....		16.45	80.0	100.0	100.00	
Casein (dog 4)	Casein (84.1%; 13.37% N)	3.15 (2.65)	10.6	13.3	19.10 (16.08)	4.86
	Lard	3.77	34.0	42.5	22.88	
	Sucrose	8.85	35.4	44.2	53.77	
	Bone ash	0.40	—	—	2.43	
	Salt mixture	0.30	—	—	1.82	
Total.....		16.47	80.0	100.0	100.00	

* Vitamin adjuvants are described elsewhere (4).

cytes, liberating globin which is as completely metabolized as dietary protein—associated with the technique of plasmapheresis would surely exert a supplementary effect erasing the amino acid deficiency in gliadin. Until this globin variable and the toxicity of high gliadin diets are eliminated, it would be theoretically and practically impossible to assay gliadin properly for the regeneration of serum protein.

For the biological assay of the proteins used in this study, the standardized plasmapheresis technique described in detail elsewhere (6, 4) was used. The object of this paper is to present the results obtained with serum protein, lactalbumin and casein together with some observations on the effect of the catabolism of tissue protein on the formation of new serum protein. The results are expressed in terms of a potency ratio defined above and based upon the fundamental concepts set forth in the first part of this paper.

The Biological Assay of Serum Protein

In the case of dog 3, it was intended to feed the increment diet immediately subsequent to the completion of the study with the serum protein diet, furnishing 8.5 per cent of the caloric intake in the form of the protein (4). Incompatibilities (7) which developed between the sera of the recipients and the cells of the donors necessitated a discontinuance of the study until satisfactory donors could be obtained. During this interval of 3 weeks, the dog subsisted on the basal protein-free diet (4) in order to prevent the accumulation of reserve serum protein stores.

When the study was resumed, dog 3 was fed the protein-free diet (period VII) and subjected to plasmapheresis to reduce the serum protein concentration to the basal level (3.5 to 4.2 per cent) and to deplete the organism of its reserve stores. Only three bleedings, resulting in the removal of 16.1 gm. of serum protein, were required to accomplish this. Evidently the protein-free dietary régime inhibited to a considerable extent the formation of new serum protein. Indeed, 10 days were required before this dog, subsisting on the serum protein increment diet, evidenced a constant and maximal response to the dietary stimulus. During this adjustment period VIII, it was necessary to withdraw 33.3 gm. of serum protein in the bleedings in order to maintain the basal level constant. In the subsequent period IX, the equilibrium week, 53.3 gm. of the blood protein were removed by plasmapheresis.

The serum protein increment diet was fed to dog 4 immediately after the study with the protein diet fed at that level normally adequate for the attainment of nitrogen equilibrium (4). Inasmuch as this increment protein was identical with the dietary protein just tested, it was not essential to deplete the animal of any carry over from the previous experimental period. During the adjustment

TABLE III

*The Biological Assay of Serum Protein for Its Value in Promoting the
Regeneration of Serum Protein*

Dog No.....	3		4	
Weight with nutritive index 0.30.....	16.70 kg.		17.30 kg.	
Diet.....	Serum protein increment		Serum protein increment	
Period.....	VIII Adjustment	IX Equilibrium	IX Adjustment	X Equilibrium
Daily intake of diet* per kg., <i>calories</i>	70	70	70	70
Average weight of dog, <i>kg</i>	16.70	16.92	17.28	17.35
Blood volume, <i>cc</i>	1105	1242	1330	1372
Plasma volume, <i>cc</i>	649	729	740	823
Average hematocrit, <i>per cent</i>	39.2	36.9	35.4	37.2
Average serum protein concentration,† <i>per cent</i>	4.20	4.75	4.82	4.94
Total bleeding, <i>cc</i>	1384	1997	2162	2454
Cell exchange				
Bled, <i>cc</i>	538	735	762	919
Injected, <i>cc</i>	657	902	1098	1183
Citrated plasma				
Citrate, <i>cc</i>	153	224	240	275
Plasma, <i>cc</i>	847	1262	1400	1535
Serum protein concentration,‡ <i>per cent</i>	3.93	4.22	4.18	4.46
Serum protein removed per week, <i>gm</i>	33.3	53.3	58.5	68.5

* The vitamin adjuvants furnished in addition 1.5 calories per kilo of body weight.

† These values were obtained by the nephelometric method as prerequisites for determining daily the size of the hemorrhages (6). The serum protein concentrations were averaged regardless of whether or not a plasmapheresis was conducted that day.

‡ An attempt (4) was made to account for the smaller serum protein concentrations in the pooled plasma aliquots as compared with the weighted average results obtained previous to the hemorrhages. The immediate entrance of tissue fluid into the circulation during the actual bleeding is responsible in part for the discrepancies noted. However, some other undetermined factor must play a rôle in causing the dilution of the plasma.

period IX, quantitative plasmapheresis resulted in the removal of 58.5 gm. of serum protein; during the equilibrium week, period X, 68.5 gm. were removed in the bleedings. The details of plasmapheresis and the nutritive histories of the dogs for the serum protein adjustment and equilibrium periods are summarized in Table III.

The Potency Ratio of Serum Protein.—

In the case of dog 3, 186 gm. of serum protein per week were essential to maintain a nitrogen balance. When 262 gm. (increment = 76 gm.) of protein were fed, 53.3 gm. of serum protein had to be removed in the bleedings per week in order to keep the concentration at the basal level. Inasmuch as 13.4 gm. of serum protein were regenerated on the basal high caloric protein-free diet (4), then 39.9 gm. must have been due to the dietary protein. However, of the dietary protein, 186 gm. must have been used to satisfy nitrogen equilibrium. Therefore, $\frac{39.9}{76} = 0.53$, the potency ratio; i.e., 53 per cent of the serum protein is utilized

to form new serum protein. In the case of dog 4, where the minimum for nitrogen equilibrium proved to be 180 gm. the potency ratio calculates as:

$$\frac{68.5 - 9.6}{92} = 0.64.$$

It is recognized that the above scheme may be criticized on the ground that the metabolism of these dogs is assumed to be the same during the evaluation of the protein minimum and during plasmapheresis. All that can be said in answer to this is that these animals throughout both studies were in an optimal nutritive state, were exposed to the same environmental conditions and were subjected to comparable feeding schedules.

The Biological Assay of Lactalbumin

During the 1st week the dogs subsisted on the protein-free diet and were subjected to plasmapheresis by which one-fourth of the blood volume was removed daily. During the subsequent week the frequency and the amount of the bleedings were determined by the daily level of the serum protein concentration. This was regarded as an equilibrium period and the values obtained were averaged with the other basal outputs for these experimental conditions (4). During the subsequent 2 weeks the lactalbumin increment diets were fed; the first week was regarded merely as the adjustment period; the subsequent week furnished the data used in the calculation of the potency ratios.

In the bleedings of the initial depletion week, a total of 43.5 and 49.1 gm. of serum protein were removed from dogs 3 and 4 respectively. The basal outputs in the following weeks were 11.2 and 4.4 gm. respectively. When subsisting on

DIET AND REGENERATION OF SERUM PROTEIN. II

TABLE IV
*The Biological Assay of Lactalbumin for Its Value in Promoting the
 Regeneration of Serum Protein*

Dog No.....	3				4			
	16.70 kg.				17.30 kg.			
	Protein-free		Lactalbumin increment		Protein-free		Lactalbumin increment	
	XII Depletion	XIII Equilib- rium	XIV Adjust- ment	XV Equilib- rium	XI Depletion	XII Equilib- rium	XIII Adjust- ment	XIV Equilib- rium
Weight with nutritive index 0.30.....								
Diet.....								
Period.....								
Average daily in- take of diet* per kg., cal- ories	101	71	70	70	94	70	70	70
Average weight of dog, kg.....	17.03	17.37	17.10	17.49	17.77	17.82	17.64	18.00
Blood volume, cc..	1315	1212	1125	1166	1305	1140	1140†	1250
Plasma volume, cc.....	725	770	671	690	735	690	690†	775
Average hemato- crit, per cent....	42.3	39.8	41.0	38.6	45.4	43.6	41.7	38.5
Average serum protein con- centration,* per cent.....	4.66‡	3.97	4.53	4.70	5.11§	3.78	4.55	4.56
Total bleeding, cc.....	1721	451	1319	1572	1956	188	1403	1526
Cell exchange Bled, cc.....	743	179	540	606	907	82	586	589
Injected, cc....	831	323	541	818	895	315	591	777

* The same qualifying statements, added as footnotes to Table III, apply also to this table.

† The blood volume for this period was not determined; the value obtained during the previous week was employed in calculating the size of the bleedings conducted during period XIII.

‡ Serum protein concentration reduced from 6.20 to 3.43 per cent.

§ Serum protein concentration reduced from 6.95 to 3.38 per cent.

TABLE IV—*Concluded*

Dog No.....	3				4			
	16.70 kg.				17.30 kg.			
	Protein-free		Lactalbumin increment		Protein-free		Lactalbumin increment	
	XII Depletion	XIII Equilibrium	XIV Adjustment	XV Equilibrium	XI Depletion	XII Equilibrium	XIII Adjustment	XIV Equilibrium
Citrated plasma								
Citrate, cc.....	194	50	146	175	216	21	156	171
Plasma, cc.....	978	272	778	964	1049	105	817	937
Serum protein concentration,* per cent.....	4.44	4.14	4.32	4.42	4.68	4.16	4.28	4.38
Serum protein removed per week, gm.....	43.5	11.2	33.6	42.6	49.1	4.4	35.0	41.1

the lactalbumin increment diet, dog 3 was able to regenerate 33.6 gm. of serum protein during the adjustment period and 42.6 gm. during the equilibrium week; for dog 4 the corresponding values are 35.0 and 41.1 gm. The details of plasma-pheresis and the nutritive histories of the dogs for these experiments are summarized in Table IV. In order to give the reader some idea of the data yielded during the previous protein-free régime, these periods have also been included in Table IV.

The Potency Ratio of Lactalbumin.—The method for calculating the potency ratio of lactalbumin is exactly the same as that employed in the case of serum protein. From the detailed calculations given below, it is evident that the results yielded by the two animals are in good agreement, the values being 0.38 and 0.34, respectively.

Dog 3.

76 gm. lactalbumin = increment above the minimum for nitrogen equilibrium of 152 gm.

42.6 gm. serum protein were regenerated during the equilibrium week.

13.4 gm. serum protein = average basal output on the protein-free diet (4).

Therefore,

$$\frac{42.6 - 13.4}{76} = 0.38, \text{ potency ratio of lactalbumin.}$$

TABLE V

The Biological Assay of Cascin for Its Value in Promoting the Regeneration of Serum Protein

Dog No.....	3				4			
Weight with nutritive index 0.30.....	16.70 kg.				17.30 kg.			
Diet.....	Protein-free		Casein increment		Protein-free		Casein increment	
Period.....	XVI Depletion	XVII Equilibrium	XVIII Adjustment	XIX Equilibrium	XV Depletion	XVI Equilibrium	XVII Adjustment	XVIII Equilibrium
Daily intake of diet* per kg., calories.....	90†	90†	70	70	90†	90†	70	70
Average weight of dog, kg.....	17.46	17.59	17.89	18.15	18.29	18.10	18.24	18.47
Blood volume, cc..	1260	1128	1143	1290	1335	1250	1250	1426
Plasma volume, cc.....	690	697	643	761	705	777	735	828
Average hematocrit, per cent.....	41.4	41.8	39.9	39.4	41.9	39.9	42.4	40.4
Average serum protein concentration,* per cent.....	4.40†	4.03	4.39	4.61	4.65§	3.80	4.20	4.49
Total bleeding, cc.....	1333	641	1310	1870	1491	432	913	1969

* The same qualifying statements, added as footnotes to Table III, apply also to this table.

† Since both dogs exhibited a decided tendency to gain in weight, the *ad libitum* consumption of the protein-free diet was discontinued, the ration being fed instead at a 90 calorie per kilo level. This slight modification in the preliminary feeding periods does not introduce any error, because the protein-free diet was still fed at the high caloric level of approximately 30 per cent above that essential for maintenance.

‡ Serum protein concentration reduced from 5.84 to 3.25 per cent.

§ Serum protein concentration reduced from 6.12 to 3.49 per cent.

TABLE V—*Concluded*

Dog No.....	3				4			
Weight with nutritive index 0.30.....	16.70 kg.				17.30 kg.			
Diet.....	Protein-free		Casein increment		Protein-free		Casein increment	
Period.....	XVI Depletion	XVII Equilib- rium	XVIII Adjust- ment	XIX Equilib- rium	XV Depletion	XVI Equilib- rium	XVII Adjust- ment	XVIII Equilib- rium
Cell exchange								
Bled, cc.....	559	267	515	736	629	169	385	793
Injected, cc.....	658	336	606	891	653	266	472	855
Citrated plasma								
Citrate, cc.....	148	70	146	209	165	48	102	219
Plasma, cc.....	774	374	795	1134	862	263	528	1176
Serum protein concentration,* per cent.....	4.33	4.20	4.24	4.18	4.41	3.81	4.14	4.14
Serum protein removed per week, gm.....	33.5	15.7	33.7	47.4	38.0	10.0	21.9	48.6

Dog 4

92 gm. lactalbumin = increment above the minimum for nitrogen equilibrium of 141 gm.

41.1 gm. serum protein were regenerated during the equilibrium week.

9.6 gm. serum protein = average basal output on the protein-free diet (4).

Therefore,

$$\frac{41.1 - 9.6}{92} = 0.34, \text{ potency ratio of lactalbumin.}$$

The Biological Assay of Casein

In the bleedings of the initial week a total of 33.5 and 38.0 gm. of serum protein were removed from dogs 3 and 4 respectively. The basal outputs in the following weeks were 15.7 gm. for dog 3 and 10.0 gm. for dog 4. When subsisting on the casein increment diet, dog 3 was able to regenerate during the adjustment period XVIII 33.7 gm. and during the equilibrium period XIX 47.4 gm. of serum protein. For dog 4 the comparable values were 21.9 and 48.6 gm. respectively. The nutritive histories of the dogs during this phase of the investigation and the details of plasmapheresis are summarized in Table V.

The Potency Ratio of Casein.—Detailed calculations of the potency ratios are given below. The results yielded by the two dogs indicate good agreement; the values are 0.45 and 0.42, respectively.

Dog 3.

76 gm. casein = increment above the minimum for nitrogen equilibrium of 186 gm.

47.4 gm. serum protein were regenerated during the equilibrium week.

13.4 gm. serum protein = average basal output on the protein-free diet (4).

Therefore,

$$\frac{47.4 - 13.4}{76} = 0.45, \text{ potency ratio of casein.}$$

Dog 4.

92 gm. casein = increment above the minimum for nitrogen equilibrium of 189 gm.

48.6 gm. serum protein were regenerated during the equilibrium week.

9.6 gm. serum protein = average basal output on the protein-free diet (4).

Therefore,

$$\frac{48.6 - 9.6}{92} = 0.42, \text{ potency ratio of casein.}$$

Effect of Tissue Protein Catabolism on Serum Protein Regeneration

Whipple and collaborators (8) have reported that 10 to 14 days are required for the fasting dog subjected to preliminary plasmapheresis to attain a normal serum protein level. Weech and associates (9) have reported a similar observation on a dog rendered hypoproteinemic by prolonged consumption of a low protein diet. Evidently tissue protein catabolism during fasting was instrumental in supplying the components essential for serum protein regeneration. Our experiment confirms this hypothesis.

Dog 5, a female collie, was fed the stock casein diet (2) until an optimal nutritive condition was attained, as evidenced by the maintenance of a satisfactory nutritive index (2). The basal protein-free diet was then administered *ad libitum*, with the animal subjected to plasmapheresis for the 1st week to deplete the body of potential serum protein stores and to reduce the serum protein concentration to the basal level. Plasmapheresis was then discontinued and the serum protein levels were determined daily in duplicate (10). The animal ate the basal diet in excess of its caloric requirement for the first 3 weeks. Subsequent refusals to eat the food voluntarily necessitated forced feeding. The quantities, thus administered, furnished each day 70 calories per kilo of body weight. After 2½ weeks of this procedure, because the animal vomited repeatedly, it was impossible to secure

retention of the food administered. The dog was allowed to fast for the remaining experimental period. The nutritive history of the dog is summarized in Table VI. In order to have a nutritive index of 0.30 the animal should weigh 11.0 kilos.

An average of the serum protein concentrations of the dog determined on 3 consecutive days, before the initial plasmapheresis was begun, was taken as the normal value and proved to be 6.75 per cent. By bleeding one-fourth of the animal's blood volume and feeding the protein-free diet, the serum protein concentration was reduced to the basal level of approximately 4.0 per cent within the 1st week (see Chart 1). With no subsequent plasmapheresis there was an immediate regeneration within the first 7 days to a maximum level of 5.16 per cent. For the next 13 days the serum protein level fluctuated near this value. Thereafter a drop occurred so that the concentration varied between 4.62 and 3.98 per cent.

TABLE VI
Nutritive History of Dog 5

Period	Duration	Daily intake of protein-free diet* per kg.	Average weight
	<i>days</i>	<i>calories</i>	<i>kg.</i>
1	8	91	10.90
2	7	72	10.67
3	7	84	10.56
4	5	37	10.31
5	18	70	10.25
6	18	0	9.21
			(Final, 8.60)

* The vitamin adjuvants (4) yielded in addition 1.5 calories per kilo of body weight.

When the animal was allowed to fast for 18 days, it was noted that after an initial delay a progressive regeneration of serum protein took place. The final value of 6.19 per cent, the average of those for the last 3 days, was for this dog somewhat below the normal of 6.75 per cent, determined 2 months previously. This increase in serum protein concentration was actually due to regeneration of the blood protein and not to hemoconcentration, as evidenced by relative plasma volumes. Chart 1 illustrates graphically the results of this study.

With dogs 3 and 4 there was observed a decided ability to regenerate serum protein during the feeding of the basal protein-free diet. However, in these animals the stimulus for regeneration was more intense inasmuch as the serum protein concentrations in these dogs were maintained at the basal level of from 3.5 to 4.2 per cent by calculated bleedings. Dog 5 also showed a similar marked tendency to regenerate above the basal level but, as the serum protein concentration rose,

the intensity of the stimulus for the formation of new serum protein decreased rapidly until at the 5.0 per cent level it was no longer manifest. This experiment emphasizes the importance of keeping the basal serum protein level as constant as possible when conducting a quantitative assay of dietary proteins by the technique of plasmapheresis.

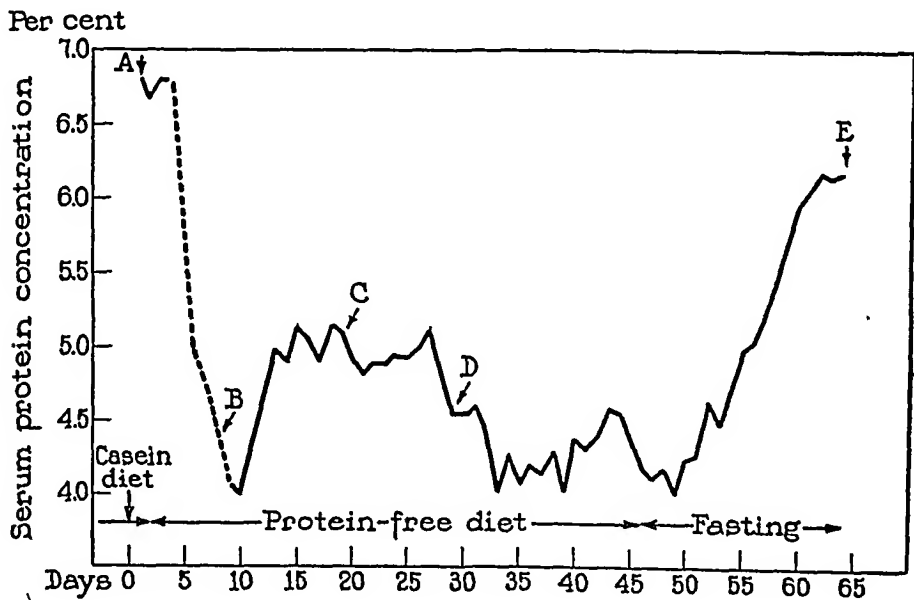


CHART 1. The influence of tissue protein catabolism upon serum protein regeneration.

-----, week of plasmapheresis.

Symbol	Blood volume	Plasma volume
	cc.	cc.
A	850	390
B	776	435
C	760	395
D	768	420
E	747	375

This study also confirms the hypothesis of Maver (11) and Bloomfield (12) that, in addition to a low protein diet, certain other variables operate in order to produce a nutritional edema.

The dormancy of the serum protein regenerating mechanism is manifest in this investigation. Thus, an animal subjected to plasmapheresis in order to deplete its serum protein concentration to the basal

level and fed the protein-free diet for as long as 45 days can still utilize tissue protein catabolites for the regeneration of serum protein.

DISCUSSION AND CONCLUSIONS

The potency ratios for serum protein, lactalbumin and casein are summarized in Table VII.

The degree of exactness of these ratio figures is somewhat uncertain because of inability to control satisfactorily the globin variable, which is described elsewhere (4). In view of this consideration these ratios do not express solely the effect of dietary protein; they probably include also the beneficial effects of any metabolized globin which was liberated from the cells laked *in vivo*. In the study with dog 4 the value of 0.64 was obtained for serum protein. This apparently high value is not inconsistent with the other ratios recorded. It is believed

TABLE VII
Regeneration of Serum Protein in Dogs Fed Artificial Synthetic Diets

Dog No.	Regenerated while on basal protein-free diet, per week	Potency ratio		
		Serum protein	Lactalbumin	Casein
3	gm. 13.4	0.53	0.38	0.45
4	9.6	0.64	0.34	0.42

to be related to the fact that during the feeding of this protein diet considerable hemolysis occurred, as a result of which it is estimated that dog 4 received in addition to dietary protein approximately 100 gm. of hemoglobin more than dog 3 during the comparable assays. If it is assumed that the true ratio value for serum protein in dog 4 has the same relation to that for dog 3 as do the values for the other proteins, then a value of 0.49 instead of 0.64 is obtained. The extent of hemolysis *in vivo* during the other protein assays in both animals was fairly comparable, judging from the cell exchanges, so that, with the value for serum protein taken as 100, the relative values of lactalbumin and casein are about 70 and 85 respectively.

The above ratings may be further questioned on the ground that no corrections can be made for the *supplementary* effects of the globin variable. In all cases the protein diets on which the dogs subsisted

when subjected to plasmapheresis contained the same absolute increments of protein above the minimum for nitrogen equilibrium. In other words, the dietary basal level was the minimal amount of the protein essential for the attainment of nitrogen equilibrium, and this varied for each of the proteins and for each of the dogs; it was the increment above this minimum which was kept constant. In the determination of the minimal amounts, the globin variable was not present, and those results are therefore valid. However, the supplementary effects of any metabolized globin, liberated from the red cell destruction *in vivo*, would probably compensate for the amino acid deficiencies thought to be responsible for the different values observed as the minima for nitrogen equilibrium. Thus, casein plus globin may be as adequate as lactalbumin plus globin for the general nitrogen requirements. Inasmuch as the animals received a *total* amount of casein greater than that of lactalbumin, a greater aliquot may have been available for the regeneration of serum protein. This may be a factor responsible for the superiority of casein to lactalbumin in promoting the regeneration of the blood protein.

In view of these criticisms of the values obtained in the present study, the results can hardly be considered as ideally quantitative. The findings do indicate, however, that serum protein is slightly superior to the others in promoting the regeneration of serum protein. Even granting that these data are subjected to error for reasons already given, the potency ratios are *comparable* and not widely divergent as those reported by others (13-15). With respect to the proteins deficient in any of the essential amino acids, determinations of their efficiency in promoting the regeneration of the blood protein have not as yet been attempted. It is obvious that in such a study, as well as in those already conducted with serum protein, lactalbumin and casein, the supplementary action of the globin variable is the most important factor tending to complicate and to invalidate somewhat the results. With regard to the clinical application of the results of this investigation, these findings suggest that all diets employed for alleviating conditions of hypoproteinemia and containing proteins in amounts appreciably more than sufficient for the attainment of nitrogen equilibrium in that individual, are probably of approximately equal therapeutic value. Certainly with respect to the proteins here

tested, the potency ratios are not so widely different as to warrant the administration of any one protein in preference to another. This conclusion is supported by the observations of Keutmann and Bassett (16), who applied the findings of Whipple and associates in feeding three patients with degenerative Bright's disease. No really distinct, significant differences were detected in the effect of the protein supplements tested. A high protein diet and a basal diet supplemented with egg white, liver protein, serum protein and lactalbumin promoted a disappearance of edema and deposition of body protein; however, the plasma protein level was unaffected.

The three complete proteins, serum protein, lactalbumin and casein, are approximately equal with respect to their value in meeting the requirements of nitrogen equilibrium. Likewise, they are of comparable value in promoting the regeneration of serum protein. In both functions a preliminary adjustment period of approximately 4 to 5 days is essential before equilibrium values are obtained. Feeding dogs subjected to plasmapheresis a protein diet at the level minimal for nitrogen equilibrium, or less, results in an impartial division of the dietary protein for the satisfaction of these two functions; the general nitrogen requirements of the organism as a whole, and serum protein formation (4). These facts indicate that serum protein regeneration is a particular phase of the general nitrogen metabolism, related to protein metabolism as a whole, and not a process with a peculiarly specific need to be met by certain particular proteins.

In the course of another investigation (1) we have obtained some experimental evidence indicating that the dog, subsisting on artificial synthetic diets and subjected to *prolonged* plasmapheresis, may suffer an impairment in its ability to regenerate serum protein. For example dog 3 under such experimental conditions was able to produce serum protein at only one-half the rate shown in the first and true biological assay of casein, although the protein diet and caloric intake remained unchanged. This inability of the animal to regenerate serum protein satisfactorily could not be attributed to the dog having previously been malnourished with the result that the dietary protein would be utilized mainly in the replenishment of wasted tissues, if body weight values can be considered as criteria. Indeed, taking the ideal nutritive index as a basis of reference, the animal was decidedly overweight and

apparently in very good health at the beginning of and throughout the study. Furthermore, it should be remembered that the casein increment diet fed to the dog contained a surplus of protein to the extent of approximately 41 per cent above the minimum required for nitrogen equilibrium. It is on account of these observations that we have emphasized giving the animals adequate rest periods subsequent to each of the biological assays. These later experiments with dog 3 together with the hypothetical evidence suggested by Bloomfield (17) and Weech and his associates (9) indicate that, even though the protein intake may be more than sufficient for the general nitrogen requirements, the serum protein regenerating capacity of the organism may fail to function to such an extent that dietary factors are no longer of distinct benefit in correcting conditions of hypoproteinemia. It would seem that in such an individual dietary protein is adequate for the general nitrogen requirements of the organism but the metabolic conversion of the tissue protein into serum protein does not operate satisfactorily. This suggests that the adequate approach to the problem of hypoproteinemia lies not so much in the evaluation of dietary factors but, as has been suggested (9), in finding a way for stimulating internally the serum protein regenerating mechanism, which seems to involve in some way the capacity of the tissues to furnish protein for the needs of the plasma. At present we are actively investigating this problem.

In a recent paper, Whipple and associates (14)⁵ state that:

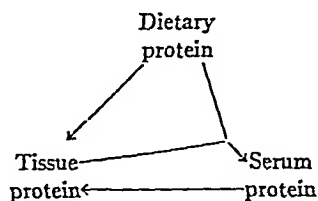
"In an emergency the plasma protein may contribute to body protein but the current will not flow easily in the opposite direction and the body proteins may be said to stand by helpless to aid while vital plasma proteins are depleted even to a lethal point. . . . Evidently in this emergency the plasma protein is very largely if not wholly dependent on materials coming in from the gastro-intestinal tract."

The evidence (14, 15) for this hypothesis is based upon the inability of their dogs, subjected to plasmapheresis, to regenerate serum protein adequately during a fasting régime. However, these investigators fail to consider that the products of tissue protein catabolism in these animals are utilized to a considerable extent simply to furnish energy, so that a limited formation of new serum protein under such circumstances is indeed not surprising. Some of their dogs did receive dextrose orally but the amounts so administered furnished each day only 21 to 24 calories per kilo of body weight with the result that the caloric requirements were

⁵ Pommerenke, Slavin, Kariher and Whipple (14), page 279.

far from satisfied, as evidenced by the pronounced losses in body weight. On purely theoretical grounds their hypothesis hardly seems tenable, for it must also involve the assumption that the ingestion of dog tissue by the dog subjected to plasmapheresis should result in no appreciable formation of new serum protein, assuming that protein catabolism, as well as protein digestion, results in the liberation of amino acids (18).

From the evidence obtained in the present work, a hypothesis is presented explaining the mechanism by which serum protein formation takes place. This is best illustrated by the following diagram:



It has already been suggested that serum protein regeneration is intimately linked with the general nitrogen metabolism of the organism. The relationship set forth in the diagram does not postulate the necessity of serum protein formation as an intermediary product in the construction of tissue protein. The assays of the three proteins, casein, lactalbumin and serum protein, differing with respect to their amino acid composition, indicated comparable potencies in promoting the regeneration of serum protein. This suggests that the absorbed end-products of protein digestion are not necessarily utilized *directly* in the formation of this blood protein, for otherwise only those proteins the chemical compositions of which show the closest resemblance to that of circulating serum protein should be decidedly beneficial for this function. If this were true, one should expect the potency ratios to vary accordingly over a wide range, which is not the case in the present study. Therefore, two mechanisms suggest themselves as being responsible for serum protein formation: (a) dietary protein may be utilized directly to form tissue protein which then may be transformed into serum protein, or (b) the absorbed end-products of protein digestion may be carried to some specific organ, the liver, for example, and there receive supplementation for any amino acid deficiencies directly by tissue protein catabolites. Mechanism (a) suggests a common source of serum protein within the body and that

this is responsible for the comparable values obtained. Mechanism (b) postulates varying degrees of protein catabolism to correspond with the extent of similarity in amino acid composition between the dietary proteins and the circulating serum protein. It is theoretically possible that both mechanisms may be in operation in the normal organism. In either case tissue protein catabolism appears to be essential in part, if not wholly, in serum protein formation.

If dietary protein is converted *exclusively* to serum protein, and if this complex subsequently is solely responsible for the formation of tissue protein, then the protein which is most potent for serum protein regeneration should also be of highest biological value with respect to its ability in satisfying the general nitrogen requirements of the organism. However, experimental findings (1) have indicated conclusively that lactalbumin is superior to serum protein in the attainment of nitrogen equilibrium, and yet this milk protein is, if anything, slightly inferior in promoting the regeneration of the blood protein. Reasoning in a similar manner, if the dietary protein is converted first to tissue protein, then those proteins which are most efficient in satisfying the nitrogen demands of the body should be most potent in promoting the regeneration of serum protein. The fact that the respective potency ratios do *not* vary directly with the values obtained from the protein minima studies indicates that serum protein can be formed in part from some other source, namely, the absorbed end-products of protein digestion after supplementation with tissue protein catabolites.

If our interpretations are correct, emphasizing the rôle of tissue protein catabolism in serum protein regeneration, then the amino acid composition of the serum protein samples, the normal, the reserve, the basal and those regenerated under the favorable dietary régimes, should be essentially the same.⁶ If these samples are quite different, then the hypothesis must be rejected.

It has been shown repeatedly with dogs 3, 4 and 4-4 that tissue protein catabolism can be *entirely* responsible for serum protein regeneration; these animals, although subsisting on a *high caloric protein-free* diet, were able to regenerate each week approximately 20 to 30 per

⁶ These determinations are being carried out as part of another study in collaboration with Dr. Richard J. Block at the New York Psychiatric Institute.

cent of the serum protein normally present in the plasma. Furthermore, the inhibitory rôle of the protein-free diet with respect to serum protein regeneration, as evidenced in the study with dog 5, was shown to be due to its effect in reducing tissue protein catabolism to a minimum. When the animal was allowed to fast, there was, after a slight delay, a progressive rise in the serum protein concentration until the normal value was approximated. In other words, during the ingestion of a protein-free diet or a fasting régime, in the course of which there can be obviously no influx of amino acids or simple polypeptides from food in the gastro-intestinal tract, serum protein regeneration occurs solely from the products of tissue protein catabolism. With the ingestion of dietary protein, the amino acid deficiencies may be supplemented directly by tissue protein catabolites, resulting in the formation of the blood protein. Only when the animal is subsisting on an adequate diet with serum protein as the only protein in the diet can we visualize the formation of the blood protein occurring independent of tissue protein catabolism.

The experiments of Osborne and Mendel (19), showing maintenance and fertility in rats fed a diet containing gliadin as its "sole source of protein," might also be interpreted as indicating a supplementary action between dietary and body protein. The increase in weight of the young showed conclusively that they were nourished satisfactorily by the mother during the 1st month of existence. Of particular interest is the observation that these lactating mothers, even though subsisting on the gliadin diet, could still synthesize casein and lactalbumin in their milk; this was probably due in part to the supplementary action of body protein, as evidenced by marked losses in body weight. A similar interpretation may be applied to the findings of Smith and Moise (20), who observed satisfactory repair of injured hepatic tissue in rats fed diets in which the deficient proteins, gliadin and gelatin, constituted practically the only source of protein.

Finally, the inability of dog 3 to regenerate serum protein adequately during the second casein assay also harmonizes with the concept that tissue protein formation may proceed independent of any intermediary formation of serum protein. This animal, fed the same protein diet at the same caloric level, exhibited a persistent gain in body weight. The initial weight at the beginning of the second assay of the protein was 18.0 kilos; the final weight, 18.9 kilos; the optimal weight of this animal to correspond with a nutritive index of 0.30 was only 16.7 kilos. However, in spite of this favorable dietary régime,

the animal was not able to regenerate serum protein adequately. Evidently the metabolic conversion of the products of protein digestion to tissue protein was not altered because the animal gained in weight; apparently some other important factor failed to operate which might well be the supplementary effect of the tissue protein on dietary protein. To test this interpretation by conducting nitrogen balance studies on dogs subjected to plasmapheresis would be practically impossible at the present time due to inability to control satisfactorily the globin variable: any urinary nitrogen values obtained would also include the nitrogen resulting from metabolized hemoglobin. Therefore, data obtained from such nitrogen balance studies would have to be considered with caution and would not warrant any satisfactory conclusions.

In the diagram illustrating our ideas of the mechanisms responsible for serum protein formation an equilibrium between tissue and serum protein is indicated. No attempt was made in the present study to obtain evidence for the conversion of the blood protein to tissue protein. However, Whipple and his associates (21) have reported that dogs receiving only sugar by mouth can be maintained practically in nitrogen equilibrium by the intravenous injections of suitable amounts of plasma, an observation which supports such a conversion.

SUMMARY

1. By the technique of quantitative plasmapheresis the effects of single proteins in artificial synthetic diets were studied with respect to their value in promoting the regeneration of serum protein.
2. The ratio of (a) the amount of serum protein per week removed by bleeding above that regenerated by the dog when eating the protein-free diet, to (b) the dietary protein increment (*i.e.*, above that required for nitrogen equilibrium) was termed the potency ratio. The results indicated that serum protein was slightly superior to casein and lactalbumin in promoting the regeneration of serum protein. However, the respective potency ratios, varying from approximately 0.51 to 0.36, were comparable and not widely divergent as those reported by others. It was concluded that, whereas in some individuals dietary proteins may be able to produce a significant increase in the serum protein concentration, the potency ratios are not sufficiently different

to warrant the administration of any one protein in preference to another.

3. The inhibitory effect of the basal protein-free diet with respect to serum protein regeneration in the dog was also demonstrated by the inability of the protein concentration to attain the normal level in spite of discontinued plasmapheresis. However, a subsequent fasting period resulted in a progressive rise in the serum protein concentration until the normal value was approximated. These observations are interpreted as indicating that the products of tissue protein catabolism can be utilized in the formation of new serum protein.

4. The experimental production of what seems to be an inhibition of the serum protein regenerating mechanism was described. This observation together with the hypothetical evidence presented by Bloomfield (17) and Weech and his associates (9) suggests that the most profitable line of approach to solution of the problem of hypoproteinemia lies not so much in the evaluation of dietary factors but in finding a way for stimulating internally the serum protein regenerating mechanism, which seems to involve in some manner the capacity of the tissue to furnish protein for the needs of the plasma.

5. A hypothesis explaining the mechanisms responsible for serum protein formation was presented and the experimental support for it discussed. The rôle of tissue protein catabolism in this function was emphasized.

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EXPERIMENTAL NEPHRITIS IN RATS INDUCED BY INJECTION OF ANTI-KIDNEY SERUM

I. PREPARATION AND IMMUNOLOGICAL STUDIES OF NEPHROTOXIN*

By JOSEPH E. SMADEL, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The etiology of the human disease, glomerulonephritis, remains unknown. Certain observations in this condition suggest that it may depend, in part at least, on a hyperergic state of the tissues. While various authors have attempted by different methods to produce experimental nephritis simulating the human affliction, only those investigations dealing with cytotoxins will be discussed. This communication presents a study of certain of the factors in the anti-organ sera responsible for the induction of renal abnormalities.

LITERATURE

Lindemann (1), in 1900, influenced by the production of hemolysins (2) and spermatolysins (3), injected guinea pigs with rabbit kidney, and obtained from these animals a serum which induced in rabbits marked albuminuria, cast formation, and tubular degeneration. Pearce (4) subsequently investigated this subject and confirmed the claims of Lindemann and others (5-7) regarding the existence of a heteronephrotoxin, but was unable to produce an autonephrotoxin (5, 7-9). He showed, moreover, (4, 10) that many of the so called specific anti-organ sera then in use depended for their effect on the hemagglutinins and hemolysins which they contained, and consequently introduced the technique of perfusing the organs used for antigens. Anti-kidney serum made with such an antigen induced albuminuria and cylindruria, whereas antiserum prepared by immunizing with unwashed kidney induced an additional feature, hematuria. The high hemolysin and hemagglutinin titer of the latter serum, he felt, was responsible for the hematuria. Even the antiserum against perfused kidney tissue was only relatively organ specific—"special" is the term he applied—since certain immune sera prepared against perfused liver induced similar kidney changes. The

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experimental nephritis was always of short duration; the pathological lesions consisted of tubular degeneration with occasional hyalin thrombi in glomerular capillaries. Woltmann (11) and Sata (12) agreed with Pearce regarding the relative specificity of nephrotoxin; the latter found that the kidney damage was less if the nephrotoxic serum were mixed with kidney emulsion before administration. Claims were made for the production of relatively specific cytotoxic sera by immunization with kidney nucleoproteids (13, 14), but not confirmed (15, 16). Wilson and Oliver (17), with anti-kidney serum free of hemagglutinins and hemolysins, produced marked glomerular as well as tubular lesions; fibrin thrombi in the glomerular capillaries and necrosis of portions of the tuft were observed; these led to scarring of glomeruli. These authors considered nephrotoxin organ specific, since absorption of the antiserum with kidney cells completely removed its nephrotoxic effect. Sakurabayashi (18) and Takeda (19) found glomerular crescent formation in the kidneys of animals followed for several months. Takeda observed fibrin thrombosis and hemorrhage in the glomerular tufts of acutely affected animals as well as tubular degeneration, and also noted an elevation of blood nitrogen values. Masugi (20, 21) recognized the anaphylactoid nature of certain of the reactions following administration of anti-kidney serum, and thought he could differentiate them from more specific cytotoxic action by absorption experiments. He believed in the relatively strict organ specificity of nephrotoxin (21-24) and also of hepatotoxins (21-23) and of cardiotoxin (25). He obtained the first convincing evidence of chronic nephritis induced by the injection of nephrotoxin, and made certain functional studies on rabbits in which he had produced this chronic disease (24). Masugi considered the experimental lesions entirely comparable to those of human glomerulonephritis (22-24). Hemprich (26), Weiss (27), and Arnott *et al.* (28) confirmed Masugi's clinical and pathological observations on rabbits treated with heteronephrotoxin.

Klinge and Knepper (29) have reopened the subject of autonephrotoxin; they injected rabbits with homologous kidney emulsion and swine serum. Fahr (30) considered the lesions they induced different from those described by Masugi (24); the latter he regarded as diffuse glomerulonephritis comparable to human nephritis.

EXPERIMENTAL

Methods

Antisera against rat tissues were prepared by injecting rabbits with suspensions of perfused rat organs. For perfusing the kidney and liver, 2 liters of 0.9 per cent NaCl solution were run into the thoracic aorta¹ at a hydrostatic pressure of 1 meter. For rendering the heart free of blood the same quantity of oxygenated warm Ringer's solution was introduced into the jugular vein at a hydrostatic level of 8 to 10 cm., the aorta was clamped just below the diaphragm and an outlet

¹ All operations were performed with the rats under ether anesthesia.

provided by snipping a tiny hole in one of the carotid arteries. The heart continued to contract throughout most of the washing. The brain was perfused by a stream of saline in the thoracic aorta directed toward the cephalic end with the ascending portion of the arch ligated. These methods rendered the desired organs blood-free in the majority of cases; when they failed, the animals were discarded.

The organs were removed, cut into fine pieces, and ground with abrasive. Saline was added to make a 10 or 20 per cent suspension. The suspensions were stored at 10°C. for 1 to 5 days. A portion of the material was cultured; those preparations giving any bacterial growth were not used. As a result of these rigorous criteria for obtaining blood-free and sterile organ suspensions, approximately 10 per cent of the material had to be discarded.

Male rabbits weighing about 2500 gm. were injected intraperitoneally with 10 cc. of a 10 or 20 per cent suspension of rat kidney, liver, heart, and brain respectively. Injections were made on 3 consecutive days each week for a period of 3 weeks. Treatment was administered for three such periods over a total of 3 months. Test bleedings were made 10 days after the last injection of each period. Antisera against rat serum and washed red blood cells were similarly prepared.

Kidney and liver emulsions were given to groups of six rabbits each, heart to four rabbits, brain to two, rat serum to three, and rat red blood cells to two.

The rabbit serum was heated at 56° for half an hour to diminish its primary toxicity. The methods used in the clinical and pathological studies on the rats are described elsewhere (31, 32). Other procedures used throughout the work will be described as the occasion arises.

Antibody Titration of Sera

Antibody titrations were made after each period of immunization, but it was found that the *in vitro* reactions bore no direct quantitative correlation to the cytotoxic action of the sera as observed *in vivo*.

Antigen for the tests was prepared by autolyzing a 20 per cent suspension of perfused organ for 12 days at 10°C. The slightly opalescent supernatant fluid was diluted to the desired concentration with saline; 0.2 cc. of the serum to be tested was added to 0.4 cc. of diluted antigen. The solutions were mixed and incubated for ½ hour at 37°C., then stored overnight at 10°C. Readings were made after each period. Rat serum precipitins were similarly determined. For titration of hemagglutinins, each tube contained 0.2 cc. of the diluted serum to be tested, 0.1 cc. of a 2 per cent suspension of washed rat erythrocytes, and 0.7 cc. of saline. In testing for hemolysins 2 units of guinea pig complement were added to a mixture of 0.2 cc. of a 5 per cent suspension of rat red cells and 0.2 cc. of the serum to be tested, then the volume was made up to 1.6 cc. The hemagglutinins and the hemolysins were recorded after incubation for ½ hour at 37°C.

Table I shows the antibody titrations, after the second immunization period, of the entire anti-kidney group of sera, and a representative serum from the anti-

heart and anti-liver groups, together with their clinical effect in rats as evidenced by urine examination and the production of edema. The two anti-kidney sera with the lowest titer of kidney precipitins failed to induce urinary abnormalities (Nos. 4195 and 4196), but two other sera (Nos. 4138 and 4197) with identical precipitin titers showed a marked quantitative difference *in vivo*.

Sera obtained after the third immunization period showed inconstant changes in test tube titrations except for a general increase in rat serum precipitins in the groups receiving kidney and heart. Nevertheless, all of the anti-kidney sera obtained at this last bleeding induced a marked effect on the kidney.

TABLE I
Antibody Titrations and Nephrotoxic Effect of Certain Anti-Organ Sera

Rabbit serum No.	Rat organ suspension injected	Tested with*						Nephrotoxic effect	
		Extract of rat			Rat serum	Rat RBC		Degree of reaction†	Dosage per 100 gm. body weight
		Kidney	Liver	Heart		Hemagglutinins	Hemolysins		
		Precipitins			Precipitins				
4138	Kidney	2400	1200	150	4800	80	160	++++	cc.
4139	"	2400	1200	150	4800	80	160	++++	0.3
4140	"	1200	1200	600	4800	40	320	++	0.5
4195	"	600	600	150	4800	40	20	0	2.0
4196	"	600	1200	150	1200	40	20	0	2.2
4197	"	2400	1200	150	4800	80	20	+	1.0
4130	Heart	400	400	400	4800	20	320	±	2.5
4124	Liver	1200	4800	150	4800	0	80	±	2.5

Sera obtained Oct. 16, 1934, after 18 injections of organ suspension.

* Precipitin titrations expressed as highest dilution of antigen giving a positive reaction; hemagglutinins and hemolysins, as dilution of serum.

† + + + + = persistent marked albuminuria and casts with anasarca.

++ = " moderate " " " without anasarca.

+ = transient " " " "

± = " slight "

Anti-Kidney Sera

Detailed reports of the functional and pathological effects which follow the administration of the nephrotoxic serum are given elsewhere (32). For the present only the more important data necessary to understand the problem at hand are described. As indicated in Table I, anti-kidney sera differed in their ability to induce renal damage. In addition, the types of lesions they induced varied, depending on the proportion of several factors in a given serum.

One factor, to which the term nephrotoxin is applied throughout this paper, affected the kidney primarily. Certain anti-kidney sera contained it in high concentration with little admixture of antibodies against other rat tissues; these were considered as "good" nephrotoxic sera. The injection of suitable amounts of such a serum was followed by marked proteinuria, casts, anasarca, and azotemia, but without significant hematuria, as revealed either macroscopically or by guaiac test. Histologically the kidney, during the acute phase of the disease, showed extensive tubular degeneration, principally of the hyalin drop-let type; but dilated tubules lined with atrophic epithelium and filled with casts were frequent. The glomeruli characteristically had thickened capillary walls visible by the routine stains; suitable stains showed that swelling of the glomerular capillary basement membrane was primarily responsible for the thickening. Degeneration of the endothelial and epithelial cells of the glomeruli was present but not severe; proliferative and infiltrative changes were practically nonexistent. When adequate, but not excessive, amounts of anti-kidney serum were given, the animals survived the acute phase. The majority continued to have protein and casts in the urine; a fair proportion, after varying intervals up to 10 months, died of the induced disease.

When a suitable amount of a good anti-kidney serum was given, a moderately severe reaction followed within a few minutes; this reaction was quite similar to the anaphylactic reaction in rats described by Parker and Parker (33) and Pratt (34). When the same serum was given in divided daily doses no such anaphylactoid reaction occurred; moreover, a slightly smaller amount of serum under these circumstances induced the same kidney effect (see Table II).

The administration of several times this optimal dose of a good anti-kidney serum in a single injection was followed by a very severe anaphylactoid reaction which sometimes killed the animal within a few minutes or hours. If the rat survived for 6 or 8 hours the kidneys showed severe acute tubular degeneration and glomerular tufts stuffed with fibrin thrombi; urine passed at this time was heavily laden with blood and protein. A few of these animals survived for a week; the hematuria disappeared in several days, the proteinuria continued, casts and anasarca appeared, and the blood urea mounted to high levels.

Anti-kidney sera rich in both nephrotoxic factor and in other anti-

TABLE II

Effect of Nephrotoxin in Single and Divided Doses

Rat N-21. One injection of serum 4138 (unabsorbed) of 0.25 cc. per 100 gm. body weight.

Date	Urine examination						Weight	Remarks	
	Albumin	Guaiac	RBC	Casts				Serum	Reaction
				Hyalin	Granular	Cellular			
1935							gm.	cc.	
Jan. 28	0	0	0	0	0	0			Control period
Feb. 2	0	0	0	0	0	0	84	0.21 iv*	Moderately severe ½ hr. duration
" 3	+++	±	0	0	0	0			
" 4	++++	0	0	0	++	0	81		
" 5	++++	0	0	0	++	0			
" 6	+++	0	0	0	+	++++	96		
" 7	+++	0	0	0	++	++	91		

Urinary findings continued approximately same until moribund on 84th day.

Rat N-140. Four daily injections of serum 4138 (unabsorbed) of 0.05 cc. per 100 gm. body weight.

Date	Urine examination							Weight	Remarks	
	Albumin	Quantitative albumin	Guaiac	RBC	Casts				Serum	Reaction
					Hyalin	Granular	Cellular			
1935		per cent						gm.	cc.	
Oct. 25	0		0	0	0	0	0	79.3		Control period
" 26	0		0	0	0	0	0	79.0		" "
" 27									0.04 iv	None
" 28	0		0	0	0	0	0	81.2	0.04 "	"
" 29	Trace		0	0	0	0	0	79.4	0.04 "	"
" 30	0		0	0	0	0	0	81.9	0.04 "	"
" 31	++	2.822	0	0	++++	+++	+++	85.3		
Nov. 1	Trace	0.489		0	++++	+++	+++	87.6		
" 2	+++	1.584	0	0	+++	+++	0	87.6		Ascites
" 4	++++	6.048	0	0	++	++	0	94.6		Subcutaneous edema and ascites

Nov. 4, 1935, killed.

* iv in all tables signifies intravenous.

bodies were obtained most frequently after long periods of immunization. Small amounts of these were capable of inducing severe kidney damage with glomerular thrombosis, hematuria, and death. Damage also occurred in other organs, notably the liver. A sublethal dose of such a serum failed to induce functional or histological renal change. To demonstrate the nephrotoxic factor in these sera it was necessary to resort to a process similar to desensitization. Beginning with a sublethal dose, at daily intervals intravenous injections of increasing amounts were given until, after 4 or 5 days, a total of 0.3 to 0.4 cc. per 100 gm. of rat body weight was injected. A clinical and pathological picture then supervened identical with that following the administration of an optimal amount of good serum.

The lethal response to injection of 0.5 cc. per 100 gm. body weight of a mixed effect anti-kidney serum is illustrated by rat N-121 in Table III. Suppression of the toxic action of the serum by administering very small doses for several days is shown by rat N-153 (Table III). The animal, after this preparation, was able to withstand an injection of 0.28 cc. per 100 gm. body weight, which was sufficient to induce the nephrotoxic effect. The mixed effect serum used in these illustrations was absorbed with rat erythrocytes and serum. As discussed elsewhere in this paper the absorptions are not followed by a reduction in the ability to induce either the anaphylactoid reaction or the nephrotoxic effect.

Clinical and anatomical studies thus indicated that anti-kidney sera possessed a factor which affected the kidney primarily and in addition contained one or more factors which induced anaphylactoid shock accompanied by a general vascular reaction. These vascular phenomena could be suppressed and the more specific nephrotoxic reaction brought out by suitable administration of serum.

Another equally effective method of eliminating the hematuria and glomerular thrombosis, while retaining the nephrotoxic effect, in such impure anti-kidney sera, was found. Rats were prepared by treating for 4 consecutive days with increasing amounts of anti-rat serum precipitins obtained from a rabbit immunized with rat serum, so that the animals finally tolerated the equivalent of several lethal doses without obvious effect. These prepared rats, when given, in a single injection, the "mixed effect" anti-kidney serum mentioned above, developed the nephrotoxic picture without hematuria. Here, as with the relatively pure nephrotoxic serum, a slightly greater amount was

TABLE III

Demonstration of Nephrotoxin in Mixed Effect Anti-Kidney Serum

Rat N-121. One injection of pooled nephrotoxic serum lot A (absorbed with rat RBC and serum), 0.05 cc. per 100 gm. body weight.

Date	Urine examination							Weight	Remarks	
	Albumin	Guaiac	Bile	RBC	Casts				Serum	Reaction
					Hyalin	Granular	Cellular			
1935								gm.	cc.	
Oct. 5	Trace	0		0	0	0	0	106.0	0.055 iv	Moderately severe ½ hr. duration
" 6	Solid	++++	+	+	0	0	0			Gross hematuria—died

Rat N-153. Four daily injections of pooled nephrotoxic serum lot A (absorbed with rat RBC and serum)—total dose 0.31 cc. per 100 gm. body weight.

Date	Urine examination							Weight	Remarks		
	Albumin	Quantitative albumin	Gusiac	Bile	RBC	Casts			Serum	Reaction	
						Hyalin	Granular				Cellular
1935		per cent							gm.	cc.	
Nov. 20	Trace	0.05	0		0	0	0	0	137.5	Control period	
" 25	0		0		0	0	0	0		0.043 iv	Slight
" 26	Trace	0.07	0	0	0	0	0	0	137.4	0.043 "	None
" 27	"	0.06	0	0	0	0	0	0	135.2	0.065 "	"
" 28	++		0		0	0	0	0		0.34 "	"
" 29	++++	3.74	0		0	++++	0	++	136.7		
" 30	Solid	5.76	0	0	0	++	+++	+++	135.9		
Dec. 2	"	5.25	0	0	0	0	++++	+++	151.8	Subcutaneous edema and ascites	

Dec. 2, 1935, killed.

Certain anti-kidney sera in small amounts, even after removal of rat hemagglutinins and serum precipitins, induce a severe anaphylactoid reaction with hematuria and death. Initial injection of a sublethal amount of such a serum followed by increasing daily doses suppresses the general vascular phenomena and brings out the nephrotoxic action.

needed if given in a single injection than when given in several divided amounts. The preparatory treatment alone failed to induce significant renal abnormalities if the initial dose of rat serum precipitins was small enough to avoid a severe anaphylactoid reaction.

Sera Prepared against Other Rat Tissues

The sera of rabbits immunized with other rat tissues were tested to determine how widespread was the nephrotoxic factor and to study individually some of the antibodies which were undoubtedly involved in the anaphylactoid reactions.

When rats were injected intravenously with adequate amounts of anti-rat serum precipitins a picture was produced indistinguishable from acute anaphylaxis. Some animals died within a few minutes, others after several hours. When the rats died within 4 to 8 hours after injection, fibrin thrombi sometimes occurred in the glomerular tuft capillaries. Hemorrhage was occasionally seen in the tufts and in the capsular space; the tubules were moderately dilated and loosely filled with albuminous precipitate. Among a number of rats injected with the same or slightly smaller amounts of anti-rat serum precipitins, some survived, and these usually developed severe hematuria and albuminuria within a few hours. These urinary abnormalities rapidly subsided (Table IV). The same phenomena have been observed after complete absorption of hemagglutinins and hemolysins from this antiserum which still contained the anti-rat serum precipitins that were considered responsible for the observed anaphylactoid reactions.

A similar type of clinical reaction has been observed after administration of small doses of hemagglutinins (Table IV). The histological findings in the kidney differed somewhat from those accompanying the simple reverse anaphylactic reaction described above, in the presence of agglutinated erythrocytes in the glomerular capillaries rather than fibrin thrombi.

When large doses of some anti-heart and anti-liver sera were given, transient albuminuria with or without hematuria was observed (Table IV). This never occurred, however, unless the serum induced a severe anaphylaxis-like reaction. Moreover, when the same total amounts of these sera were given in several divided doses at daily intervals, the shock, albuminuria, and hematuria did not occur.

Two rabbits that received injections of rat brain had at the end of the second period of immunization no demonstrable nephrotoxin in their sera. One was discarded, the other kept for 6 months, when two more series of injections were given. The method was altered: The rabbit during the third series received 1 or 2 cc. of 10 per cent emulsion of rat brain intracutaneously along with sufficient staphylo toxin to produce good erythema of the site. During the fourth series the brain emulsion and staphylo toxin were given intravenously. The serum obtained after this series of injections had some nephrotoxic effect; its action, however, resembled that of a weak mixed effect anti-kidney serum (see Table V).

TABLE IV

Effect of Sublethal Amounts of Rat Serum Precipitins
 Rat S-5. One injection of 2.5 cc. of serum 4142 per 100 gm. body weight
 (precipitin titer for rat serum 1:20,000).

serum 4142 per 100 gm. body weight						
Date	Urine examination			Weight gm.	Remarks	
	Albumin	Gua'ac	Microscopic		Serum	Reaction
					cc.	
1934						
Dec. 7	0					
" 9	Trace	0	Normal			
		0	"			
" 10	+++					
" 11	++	++++	Few RBC	58.0	1.35 iv	Control period
		0	Occasional			Moderately severe
" 12	Trace		granular cast			½ hr. duration
" 13	0	0	No cast			Gross hematuria
		0	" "			

Effect of Sublethal Amounts of Rat Hematoxins

Rat A-5. One injection of 0.1 cc. of serum 4142 per 100 gm. body weight.

Agglutination for 10 min.

Effect of Sublethal Amounts of Rat Hemagglutinins
 Rat A-5. One injection of 0.1 cc. of serum 4148 per 100 gm. body weight
 (agglutination for rat RBC > 1:800).

RBC > 1:800).								serum 4148 per 100 gm. body weight	
Date	Urine examination				Weight	Remarks			
	Albumin	Guaiac	Bile	Microscopic		Serum	Reaction		
1935									
Mar. 20	Trace	0	0	Normal	gm. 213.0	cc. 0.21 iv	Very severe $\frac{3}{4}$ hr. duration Gross hematuria		
" 21	++	++++	0	Few RBC					
" 22	Trace	0	++	Normal					
" 23	"	0	++	"					

Rat C-5. One injection of 2.5 cc. *Effect of Anti-Rat Heart Serum*

Effect of Anti-Rat Heart Serum
 Rat C-5. One injection of 2.5 cc. of serum 4130* per 100 gm. body weight.

Heart Serum						
Injection of 2.5 cc. of serum 4130* per 100 gm. body weight						
Date	Urine examination			Weight gm.	Remarks	
	Albumin	Guaiac	Microscopic		Serum cc.	Reaction
1934						
Nov. 29	Trace	0	Normal	91.4	2.3 iv	Control period Moderately severe
" 30	"	0				
Dec. 1	+	+				
" 2	+	+				
" 3	+	0				
" 4	+	0				
" 5	Trace	0				
" 6	0	0				

See Table I for antibody titer.

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See Table I for antibody titer.

TABLE V
Effect of Anti-Rat Brain Serum

Rat B-12. One injection of anti-rat brain serum 4150, 0.5 cc. per 100 gm body weight.

Date	Urine examination						Weight	Remarks	
	Cubic centi- meters	Albumin	Quantitative albumin per cent	Guaiac	Microscopic	Serum		Reaction	
1935						gm.	cc.		
Aug. 23	1.0	Trace		0	Normal	123.6			
" 27	1.0	0			"	132.6			
" 28	2.0	+	0.25	++	Rare RBC	124.4	0.65 iv	Control period	
" 29	1.5	++	1.6	+	Normal	124.2		Moderately severe	
" 31	1.0	+++	2.7	0	Few granular casts	122.3		1/2 hr. duration	
Sept. 2	2.5	+++	2.01		Many hyalin and granular casts	122.3		Urine straw-colored —diarrhea	
" 6	6.0	+	0.38		No casts	130.3			
" 8	2.5	++	1.03		Few casts	135.7			
" 10	1.5	+	0.31	0	No casts	124.9			
" 12	1.5	Trace	0.86	0		128.0			
Rat B-18. Injection of 1.7 cc. serum 4150 (6 doses, per 100 gm. body weight)							Chloroformed		

Rat B-18. Injection of 1.7 cc. serum 4150 (absorbed with RBC) in divided doses, per 100 gm. body weight.

1935 (absorbed with RBC) in divide									
Date	Cubic centi- meters	Albumin	Urine examination			Weight	Remarks		
			Quantitative albumin	Guaiac	Microscopic		Serum	Reaction	
									per cent
1935						gm.	cc.		
Oct. 2	1.0	Trace			Normal	89.0			
" 3	0.1	0			"	85.1		Control period	
" 4	0.1	0			"	84.5		"	
" 5	0.5	Trace			"	88.0	0.17 iv	Slight	
" 6	0.8	"	0		"		0.18 "	None	
" 7	2.0	"	0		"	90.6	0.36 "	"	
" 8	1.0	"	0		"	91.8	0.8 "	"	
" 9	8.0	++	0.46		"	92.9			
" 10	0.4	++	1.5		"	94.8			
" 12	2.5	+++	0			104.0		Slight ascites	
" 13	1.8	++				111.3		Ascites	
" 14	1.5	++++	2.5	0	Many hyalin, granular, and cellular casts			Ascites. Chloro- formed	

To recapitulate, the criteria we have chosen for establishing the presence of rat nephrotoxin in an antiserum, namely, the ability to induce on injection into rats marked persistent albuminuria and cylindruria, ascites, and elevated blood urea, but without hematuria, have been fulfilled only in sera from those animals immunized with rat kidney or brain. The criteria have not been fulfilled by rabbit sera prepared against rat heart, liver, serum, or red blood cells. The occurrence of nephrotoxin, even though in rather small amounts, in one anti-rat brain serum suggests that it is only relatively organ specific.

Toxic Effect of Certain Diluents

Several observations suggested that an adventitious toxic factor not associated with serum, acting in conjunction with an optimal or suboptimal dose of nephrotoxin, might induce hematuria or glomerular thrombosis, or both.

In early experiments, in attempting to increase the accuracy of administration of small amounts of serum, the serum was diluted with one or two volumes of Ringer's solution. This did not affect the nephrotoxic action; but when one particular lot of Ringer's solution was used, hematuria occurred. This solution was approximately 6 weeks old when first used, was sterile, and in amounts of 5 cc. intravenously by itself was harmless. 40 rats received initial intravenous injections of 0.05 to 0.3 cc. per 100 gm. body weight of serum 4138. Among seven that received serum diluted with the Ringer's solution under discussion, four developed hematuria. None of the 33 rats which received optimal or suboptimal doses of this serum, administered either undiluted or with other lots of Ringer's solution, showed hematuria.

Attempts to Transmit the Induced Disease

Sera obtained from rats in the acute or terminal stages of this type of experimental nephritis have been injected intravenously into five rats in amounts varying from 0.65 to 6.9 cc. per 100 gm. body weight, without inducing any evidence of renal damage.

Effect of Rat Nephrotoxin in Other Animals

Nephrotoxic serum 4138 has been given to a small number of mice and guinea pigs. In mice the dosage varied from 0.35 to 2.0 cc. per 100 gm. of body weight; in guinea pigs 0.9 cc. and 1.0 cc. per 100 gm. were given. In none of these animals was there significant patho-

logical change, nor was any abnormality observed in the guinea pig urine.

Absorption of Anti-Kidney Sera

Absorption with Kidney and Liver Cells.—Further information concerning the specificity of the factors in the anti-kidney serum responsible for the two disease pictures was obtained by absorption with various substances. Nephrotoxic sera when absorbed with washed cell suspension of rat kidney and rat liver completely lost their ability to induce kidney damage and the anaphylaxis-like reaction was almost entirely eliminated.

After exsanguination, the kidneys of two adult rats were aseptically removed. The four kidneys, having a combined weight of approximately 4 gm., were finely minced and ground with an abrasive. The material was suspended in 250 cc. of saline, centrifuged, and the supernatant fluid was discarded. Two more washings resulted in a clear supernatant fluid. The sediment was suspended in 5 cc. of saline and centrifuged at high speed. This gave a three layer zoning; coarse particles, a tan colored thick suspension of very fine particles, and a clear upper zone. The midzone was removed and again centrifuged to remove as much excess fluid as possible. 4 cc. of anti-kidney serum 4138 were added to the sediment of kidney cells, thoroughly mixed, incubated overnight at 10°C., and then the cells were removed. The entire procedure was carried out three times; thus cells from 12 gm. of kidney were used. The precipitin titer against autolyzed and fresh kidney antigen decreased with each absorption until, after the third, the serum gave only a faint reaction with an antigen dilution of 1:240.

A second lot of 4 cc. of this nephrotoxic serum was similarly absorbed with cells from a total of 40 gm. of liver. The suspension of cells obtained from one liver (approximately 8 gm.) was used in the first absorption, but on the next two occasions the cells from two livers were used. Precipitins against rat liver decreased during the absorption, and in the end were faintly positive at a dilution of 1:240. The nephrotoxic serum absorbed with washed kidney cells was tested in three rats, and that absorbed with liver in nine. In each instance, 0.3 cc. per 100 gm. body weight, an optimal dose of the unabsorbed serum was given intravenously and repeated on the 2nd or 4th day. No significant urinary change developed over periods of observation up to 6 months, with one exception. This animal, which received the liver-absorbed serum, ran a normal course for 2½ months, then developed proteinuria and casts after receiving a skin test with kidney autolysate.

Attempts to repeat this observation failed.

Absorption with Fat-Free Kidney Suspensions.—The nephrotoxic factor is readily removed by absorption with fat-free kidney suspension.

The lipid-free tissue suspension was prepared as follows: Perfused rat kidneys were removed, minced, and ground with abrasive; ten volumes of cold absolute alcohol were added. The mixture was frequently shaken while kept at 0°C. for an hour; the sediment obtained by centrifugation was again resuspended in cold absolute alcohol and allowed to extract for 2 hours. The sediment was then subjected to two extractions with anhydrous ethyl ether, the final one lasting overnight. The sediment was dried under vacuum and sealed until used.

The absorption was carried out as follows: Dried fat-free kidney powder, obtained from 4 gm. of kidney (wet weight) was triturated with enough saline solution to make a thick paste. This was transferred to a tube and centrifuged at high speed. The small amount of supernatant fluid was discarded. 2 cc. of pooled mixed effect anti-kidney serum were added to this sediment, thoroughly mixed, and incubated at 37°C. for 1 hour and at 10°C. overnight; 2.1 cc. of absorbed serum were recovered.

The serum, before absorption, induced the typical nephrotoxic picture when 0.4 cc. per 100 gm. body weight was given in divided doses. After absorption, 0.15 cc. per 100 gm. body weight in a single injection resulted in a fatal anaphylactoid reaction, but 0.4 cc. and 0.65 cc. given in divided doses failed to produce any significant functional or pathological damage. The same absorption technique was applied to another portion of the serum; 0.72 cc. and 0.8 cc. of this in divided doses also failed to induce an effect on the kidney. 2 cc. of an anti-rat kidney serum, capable of inducing a typical nephrotoxic effect when 0.4 cc. per 100 gm. body weight is injected into rats, contain enough nephrotoxin to affect roughly 6 gm. of kidney, since the combined weight of the kidneys of a 100 gm. rat is about 1.2 gm. After absorption of 2.0 cc. of a serum of such potency with the fat-free kidney fraction from 4 gm. of kidney, the nephrotoxic activity was very markedly reduced.

Absorption with Fat-Free Liver Suspensions.—

By the same procedures fat-free liver tissue was prepared. Much larger amounts of liver tissue were employed in this experiment; five times the proportion of tissue used in the kidney absorption was added for the first liver absorption, yet this removed only a fraction of the nephrotoxin. An amount equal to an optimal dose of unabsorbed serum induced a moderate proteinuria and cylindruria, and one and a half times this dose produced a typical effect. When the remainder of this partially absorbed serum was treated with the same quantity of fat-free liver, no nephrotoxic factor was demonstrable.

These experiments indicated that while fat-free liver suspensions were capable of removing the nephrotoxic factor, relatively large amounts in comparison with fat-free kidney were required. The quantities of liver cell and fat-free liver suspensions necessary to absorb the nephrotoxin were roughly comparable.

Absorption with Alcohol-Ether-Soluble Fraction of Kidney.—The nephrotoxic factor was not appreciably affected in sera treated with alcohol-ether-soluble fraction of kidney.

The alcohol and ether extraction fractions used in rendering 12 gm. of kidney fat-free were pooled and distilled to dryness under vacuum, at 45°C. Alcohol-ether mixture 3:1 dissolved most of the residue; the soluble portion was stored at ice box temperature until needed. A portion of the material representing the soluble fraction from 5 gm. of kidney was evaporated to dryness at room temperature under vacuum. 2 cc. of pooled anti-kidney serum, of the same lot as used in the experiments just described, were added to the dry residue and triturated. A gummy emulsion with curds resulted, but after warming at 37° for half an hour, it became a creamy homogeneous emulsion. This was further refrigerated overnight at 10°C. Centrifugation at 5000 R.P.M. for 10 minutes stratified the mixture into a slight finely granular precipitate, a faintly hazy solution, and a fatty crust. The midlayer was removed with a capillary pipette.

As mentioned above, 0.4 cc. of this pooled serum in divided amounts was sufficient to induce a typical nephrotoxic picture; after absorption the same amount still produced the effect. In addition, the anaphylactoid-inducing properties of the serum were still present; 0.4 cc. in a single injection killed the rat in 4 hours. The kidneys showed fibrin thrombi in the glomerular tufts. On another occasion the alcohol-ether-soluble fraction from 5 gm. of kidney was added to a similar amount (2.0 cc.) of serum. In this experiment the slightly hazy absorbed serum obtained after centrifugation was passed through a Seitz filter with resulting clarification. This specimen lost some of its potency during treatment, as 0.6 cc. in divided doses was necessary to induce a severe acute stage of the disease. In filtering small amounts of nephrotoxic serum it was quite important to prepare the filter properly in order to prevent loss of activity, loss of volume, or dilutions of serum. By first passing 5.0 cc. of broth, followed by 4.0 cc. of normal rabbit serum, through the pad, it was possible subsequently to filter 4 or 5 cc. of nephrotoxic serum without appreciable loss of potency, but when only 2 cc. were filtered some loss of activity usually occurred.

Absorption with Petrol-Ether-Soluble Lipids.—Petrol-ether-soluble lipids were obtained from perfused rat kidney, liver, brain, and abdominal fat.

An alcohol-ether extract of each tissue was prepared by the method described above; this was evaporated to dryness under vacuum at 45°C. That portion of the residue which was soluble in petrol-ether was extracted. The petrol-ether containing the lipids was concentrated to a small volume by evaporation and stored at 10°C. Anti-kidney serum was absorbed by the same methods as employed for the alcohol-ether-soluble fraction, with clarification by passage through a properly

prepared Seitz filter. 174 mg. of each lipid were added to separate samples of 4.5 cc. each of serum 4139, which was capable of inducing a severe acute disease in single doses of 0.4 cc. per 100 gm. body weight.

The results of these experiments were entirely negative; none of the lipids removed a significant amount of nephrotoxin or anaphylactoid-inducing factor from this serum.

Removal of Hemagglutinins and Hemolysins.—Absorption of nephrotoxic serum with red blood cells did not alter its optimal dosage.

To 3 cc. of serum 4138, were added 2 cc. of washed packed rat erythrocytes. The mixture was placed for 2 hours in the ice box and centrifuged in the cold. This serum, which then failed to show either hemagglutinins or hemolysins in dilutions of 1:2.5, had lost none of its kidney-damaging properties.

Removal of Anti-Rat Serum Precipitins.—Removal of anti-rat serum precipitins from anti-kidney serum did not diminish its nephrotoxic activity.

10 cc. of each of the four mixed effect anti-kidney sera obtained after the third period of immunization were pooled. The mixture was found to induce the standard acute disease when 0.4 cc. was given in divided doses. This pooled serum had a hemagglutinin titer of 1:30, and serum precipitin titer of 1:200,000. A 30 cc. portion was first treated with 9 cc. of packed washed rat erythrocytes, which reduced the hemagglutinin titer to \pm at 1:5 dilution. Next 0.03 cc. of normal rat serum was added, stored overnight at 10°C., and centrifuged at 0°C. After repeating the last procedure, the anti-rat serum precipitin titer was \pm at 1:100 dilution.

The absorbed portion simulated the unabsorbed in its induction of the nephrotoxic lesions, the dosage being identical in both instances. Slightly less of the absorbed serum, on the initial injection, induced the nonspecific general vascular phenomena; the anaphylactoid reaction in this case usually became severe several hours after injection, and death occurred in 12 to 18 hours. The animals presented in Table III received injections of the absorbed portion of this pooled anti-kidney serum.

The absorption experiments summarized in Table VI demonstrated that the nephrotoxic factor was not related to the antibodies against red blood cells or serum, that it was removed by contact with kidney and liver cells, that fat-free kidney in amounts comparable to the tissue affected *in vivo* readily withdrew it from serum, while fat-free

liver did so when large quantities were used, and that the rat lipids did not remove it. This evidence suggests that nephrotoxin, while not strictly organ specific, might be considered as relatively organ specific.

Fractionation of Nephrotoxic Serum.—The evidence so far presented indicated that the nephrotoxic factor was a relatively specific antibody for kidney. It was felt that its presence, if demonstrable, in the globulin fraction of the serum, would lend additional support to the conception of its antibody nature.

Pooled nephrotoxic serum absorbed with erythrocytes and rat serum was fractionated in the following manner: To 4 cc. of the serum was added an equal amount of saline, followed by 8 cc. of saturated ammonium sulfate solution. The

TABLE VI
Absorption of Nephrotoxic Agent from Anti-Kidney Serum

Absorbing material	Removal of nephrotoxin
1. Kidney cell suspension	Complete
2. Fat-free kidney tissue	"
3. Alcohol-ether-soluble kidney extract	Very slight
4. Petrol-ether-soluble kidney extract	None
5. Liver cell suspension	Nearly complete
6. Fat-free liver tissue	" "
7. Red blood cells	None
8. Serum	"

mixture was shaken for 15 minutes, then centrifuged, the globulin precipitate was taken up in 5 cc. of $M/100$ phosphate buffer solution. Crystalline $(NH_4)_2SO_4$ was added to the supernatant until saturation was reached; the albumin precipitate was obtained by centrifugation, then dissolved in buffer solution. Both fractions were dialyzed, in collodion sacks, at ice box temperature, against frequently changed phosphate buffer solution. After 18 hours the dialysates no longer gave a positive reaction for sulfate ions. On removal from the sacks sufficient dry NaCl was added to bring the concentration to 0.1 per cent.

The nephrotoxin, as well as the anaphylactoid-inducing substance, was recovered quantitatively from the globulin fraction. The albumin fraction was innocuous.

Effect of Freezing and Drying.—Anti-kidney serum was frozen in a carbon dioxide ice and alcohol mixture and dried, while in the frozen

state, by the method of Swift (35) for preservation of bacteria. When the desiccated serum was taken up in distilled water it still retained its properties of inducing the nephrotoxic effect and the anaphylactoid reaction.

DISCUSSION

The present experiments demonstrate that anti-kidney serum, prepared by immunizing rabbits with perfused rat kidney, is toxic when injected into rats and is capable of inducing profound kidney changes. Several different antibodies are responsible for these disturbances; a relatively organ specific antibody against kidney is present, as well as non-organ specific antibodies against various rat tissues, serum, and erythrocytes. A number of the non-organ specific antibodies, when injected in proper amounts, induce a shock-like reaction with accompanying lesions throughout the body. The clinical and pathological derangement of the kidney observed after administration of these antibodies differs significantly from those occurring after treatment with the relatively organ specific antibody nephrotoxin.

It is not surprising that rabbits immunized with a suspension of perfused rat kidney should produce antibodies against rat tissues other than kidney. Although the perfusion seemed complete, there were undoubtedly still present in the organ suspension sufficient erythrocytes and serum to stimulate some antibody formation. In addition, the antigen suspensions contained tissues that occur in other organs—smooth muscle, endothelium, and fat. The presence of these in the different organ extracts used in the precipitin tests might account for some cross reactions. Furthermore, the cell constituents peculiar to the animal species probably call forth antibodies which react to some extent with any antigenic substance from that species. Fleisher (36) has emphasized the complexity of tissue antigens. The failure in this work to correlate the nephrotoxic activity of anti-kidney serum with its precipitin titer for kidney extracts agrees with the observation of Pearce (4), but is contrary to that of Masugi (23). Since the antigenic fraction, or fractions, of kidney responsible for the production of nephrotoxin are not at present known, and hence cannot be isolated and tested *in vitro*, only biological assay remains as the means of determining the amount of nephrotoxin in an antiserum.

In the present experiments, the reaction believed to result from nephrotoxin alone was differentiated from the combined effect of nephrotoxin and severe anaphylactoid response. In the former hematuria and fibrin thrombosis of glomerular capillaries did not occur, while they were prominent in the latter. This leads to a reaffirmation of the statement of Pearce (4), "In determining the injury inflicted by nephrotoxin upon the kidney parenchyma, hemaglobinuria must be avoided." The concept of Pearce, however, that hemagglutinins and hemolysins occurring in the anti-kidney serum were solely responsible for the hematuria, must be amplified to include other antibodies against rat tissues, and even toxic substances in solvents. This admonition to avoid hematuria has gone practically unheeded—in fact, most workers have emphasized the presence of erythrocytes in the urine of treated animals, and considered this a point of similarity between the experimental and human nephritis.

It has been shown that the severe anaphylactoid reaction, which results in damage to most of the viscera, could be prevented by a process of desensitization; the nephrotoxic effect, on the other hand, was not inhibited by these procedures. Whether this difference between nephrotoxin and other tissue antibodies is only quantitative, the evidence at hand was insufficient to determine. The inability to desensitize readily against the nephrotoxic effect by procedures which eliminate not only the general vascular phenomena of the anaphylactoid reaction, but also the focal necrosis of liver cells (such as described by Masugi (23) as a characteristic effect of hepatotoxin) which is observed after injection of certain mixed effect anti-kidney sera, suggests that the nephrotoxic reaction differs basically from the more common hyperergic reaction in tissue. The possibility that the nephrotoxic effect is dependent on an anaphylatoxin developing from the union of any rat tissue and appropriate antibody seems remote. For, theoretically, appreciable quantities of anti-rat serum precipitins produced by injecting large amounts of anti-rat serum should be into rats. This can be done without inducing a discernible anaphylactoid reaction if daily increasing doses of anti-rat serum are given, beginning with a small injection. The failure to induce the nephrotoxic picture by this method was described.

Several earlier workers (4-6, 8) were able to induce albuminuria and casts by injecting serum from an animal with nephritis, produced

experimentally by the injection of nephrotoxin, into a second animal of the same species. These observations were made on dogs and rabbits. In Pearce's hands the phenomenon was observed in about half of the experimental dogs and was never severe. Rats, used in the present work, did not respond in this manner when given serum from rats with nephrotoxic nephritis. Whether this indicates a difference in reactivity of the two animal species or whether the earlier observations were dependent upon the injection of serum from an animal of a different blood group is uncertain.

The complete absorption of nephrotoxin by kidney suspensions has been previously recorded by Wilson and Oliver (17), and by Masugi (21). The latter also carried out the procedure with liver suspensions but did not find this substance effective in removing the nephrotoxic agent. His results, given in abstract form, without protocols, apparently differ from those cited here where liver was found to be much less effective than kidney, but nevertheless capable of completely removing nephrotoxin from anti-kidney serum if used repeatedly.

It hardly seems necessary to discuss the complete independence of nephrotoxin and the antibodies against red blood cells and serum. The failure of the latter antigens to produce nephrotoxin when used for immunization, or to absorb it when present, agrees with the experience of most workers.

The failure to remove the nephrotoxic effect of anti-kidney serum by absorption with lipidal elements of kidney, but its complete absorption by fat-free kidney substance was described in the present experiments. This differs from the conclusions of Hahn (37), who tested anti-kidney serum entirely *in vitro*.

Nephrotoxin, while not strictly organ specific, is undoubtedly relatively specific for kidney. It occasionally occurs in antisera, in small amounts, after immunization with tissues other than kidney, and may, with some difficulty, be absorbed from antiserum by liver suspensions. Nephrotoxin, however, affects the kidney principally, appears most regularly and in highest titer after immunization with kidney tissue, and is most easily absorbed from nephrotoxic antiserum by kidney tissue.

SUMMARY

Nephritis can be induced in rats by the injection of anti-kidney sera obtained from rabbits immunized with suspensions of perfused rat kidney.

Anti-kidney sera, thus prepared, contain a number of antibodies capable, on injection into rats, of inducing a severe anaphylactoid reaction with general vascular manifestations that involve the kidney as well as other organs. These sera also contain a nephrotoxic agent that affects the kidney primarily.

The nephrotoxic effect is characterized clinically by severe persistent albuminuria with casts, and transient anasarca during the acute disease, but no significant hematuria occurs. When a severe anaphylactoid reaction is superimposed on the nephrotoxic injury, hematuria is an outstanding feature.

Nephrotoxin is demonstrable *in vivo* and is not related quantitatively to the precipitins in the anti-kidney serum against kidney extract. It is most readily obtained by immunization with kidney suspensions, but may occasionally appear after injections of other organ preparations; it does not result from immunization with erythrocytes or serum. Nephrotoxin is present in the globulin fraction of anti-kidney serum.

The nephrotoxic action of anti-kidney serum is easily removed by absorption with kidney cells or fat-free kidney tissue. Similar preparations of liver likewise remove it, but less readily. Neither kidney, liver, or brain lipids affect it, nor does absorption with red blood cells or serum.

Nephrotoxin appears to be an antibody that is relatively organ specific in its affinities. It differs from the more common antibodies involved in reverse anaphylaxis in one respect, at least: The animal rapidly becomes desensitized against the latter and fails to react, whereas desensitization to nephrotoxin is difficult to secure.

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TISSUE CULTURE STUDIES ON BACTERIAL HYPERSENSITIVITY

III. THE PERSISTENCE IN VITRO OF THE INHERENT SENSITIVITY TO TUBERCULIN OF CELLS FROM TUBERCULOUS ANIMALS

By JOHANNES K. MOEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

PLATES 49 AND 50

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Sensitization of mesenchymal cells to tuberculin occurs regularly during the course of tuberculous infections in guinea pigs; this can be shown by the specific toxic action of tuberculin acting on these cells in tissue cultures (1). Available evidence indicates that the sensitivity is an inherent characteristic of the cell; and this opinion was strengthened by the demonstration that the characteristic sensitivity persisted in tissue cultures that had been transplanted several times (1). In order to eliminate the possibility that tissue fluids, carried over with the explants, may have been responsible for the persistence of the cellular sensitivity *in vitro*, it seemed desirable to determine whether growths derived from single cells, or from small aggregates of cells, and comparatively free from tissue fluids of the tuberculous environment, would retain their hypothetical inherent sensitive state.

Studies by others show that leucocytes from tuberculin sensitive patients and from tuberculous animals, exhibit increased susceptibility to the toxic action of tuberculin. Holst (2) found that tuberculin was highly toxic for leucocytes from sensitive animals, and that it greatly inhibited the phagocytic activity of these cells. Supravital studies by Stewart, Long, and Bradley (3) of exudative cells, obtained by injecting intrapleurally broth containing tuberculin into tuberculous animals, showed early death of all cellular elements. Similar cells obtained in like manner from normal animals were apparently unaffected by the tuberculin.

In a previous study (4) it was shown that an almost pure mono-

nuclear exudate could be produced by the intrapleural injection of molten parowax, a commercial wax for household uses. Most of these cells when explanted into tissue culture media, behaved as migrating macrophages or wandering cells; a smaller number transformed into morphologically typical fibroblasts which proliferated to form macroscopic colonies. It seemed, therefore, that by using this technique, the criteria of obtaining single or small aggregates of cells as free as possible from body fluids could be achieved; and the question of the inherent sensitivity to tuberculin could be more decisively answered.

EXPERIMENTAL

Mononuclear exudative cells were produced by the intrapleural injection of 0.5 cc. of molten parowax into normal guinea pigs and into animals infected intratesticularly with 5.0 mg. of the lowly virulent strain R 1 tubercle bacillus for 3, 4, and 5 weeks respectively. 5 to 7 days later very light suspensions of mononuclear cells were obtained by washing the exposed pleural cavities of the animals with 2.0 cc. of Tyrode's solution. The technique for obtaining these cells with a minimum of trauma and practically free of admixed erythrocytes has been fully described (4). The injection of parowax as a pleural irritant or incitant of mononuclear exudative cells was associated with the production of only very small amounts of fluid exudate. This body fluid was greatly diluted by the irrigation of the pleural cavity with Tyrode's solution, and was further diluted 15 times when the cellular suspension was added to the culture media. Thus the concentration of body fluids in contact with the cells in the culture flasks would seem negligible.

0.1 cc. of the cellular suspension, consisting mostly of single cells with few microscopic clumps of cells, was explanted to Carrel micro flasks containing 0.75 cc. normal guinea pig plasma, 0.15 cc. diluted tuberculin solution, and 0.5 cc. of 10 per cent guinea pig splenic extract. The plasma and tissue extract were prepared as previously described (1). The various portions of the media were thoroughly mixed while in the fluid state to distribute the mononuclear cells uniformly. Coagulation of the media soon followed. The cultures were incubated at 37.5°C. A typical tissue culture experiment consisted of mononuclear exudative cells from a tuberculous animal in flasks containing: (a) old tuberculin in a concentration of 1 to 300, (b) old tuberculin in a concentration of 1 to 600, and (c) normal media. A like number of flasks were employed for testing the exudative cells from a normal animal.

The comparative cytotoxic index of tuberculin on splenic cells from the same tuberculous and normal guinea pigs was determined as previously described (1). The fibroblastic growths resulting from transformation of mononuclear exudative cells were transplanted by removing the fibrin clot from the flask, excising the growths, dividing them into equal sized transplants, which were then transferred into flasks containing media with and without tuberculin. Cultures of fibroblasts

were renourished every 3 or 4 days with 0.5 cc. of 5 per cent guinea pig splenic extract; tuberculin, in the same concentration as in the coagulum, was added to those flasks containing that substance. At times, small amounts of 25 per cent guinea pig embryonic extract were added as a growth stimulant. Quantitative measurements of fibroblastic growth were made by the projectoscopic and planimetric method (5). The comparative cytotoxic index of tuberculin for sensitive cells was determined by the method described previously (1).

RESULTS

Supravital Study of Mononuclear Exudative Cellular Suspensions.—

The cellular suspensions obtained from both normal and tuberculous animals consisted almost entirely of mononuclear cells, lymphocytes, monocytes, and clasmotocytes. The cells were mostly isolated, with only occasional small clumps of microscopic size. The number of cells per unit volume of suspension from normal and tuberculous animals was essentially the same. Very few red blood cells were present.

Growth of Mononuclear Cells in Normal Media.—Most of the single isolated cells and many of the cells in clumps developed as typical migrating macrophages with long protoplasmic processes, pseudopodia, and undulating membranes. A few of the isolated cells transformed into typical fibroblasts, many of which proliferated to form small colonies. Luxuriant growths of fibroblasts developed from some of the microscopic clumps of cells, so that by the end of a week comparatively large macroscopic growths were visible. The type of growth and rate of proliferation of mononuclear cells *in vitro* is more fully described elsewhere (4). The types of cells and extent of proliferation of mononuclear cells from the tuberculous and the normal animal were essentially the same when grown in normal media.

Growth of Mononuclear Cells in Media Containing Tuberculin.—Mononuclear cells from the normal animal were only slightly inhibited by the concentration of old tuberculin used (1 to 300 and 1 to 600); injury was indicated by slightly increased granulation of the cytoplasm, smaller cellular size, and attenuated protoplasmic processes. Fibroblastic proliferation appeared slightly inhibited in media containing old tuberculin 1 to 300; there was very little, if any, inhibition of fibroblastic growth from normal cells in media containing old tuberculin 1 to 600.

Mononuclear cells from the tuberculous animal, on the other hand,

were distinctly inhibited by old tuberculin in the two concentrations used. Most of the cells were small, round, dark, and appeared inactive or dead. Fibroblastic forms were also greatly inhibited. They were dark in color, heavily granulated, and swollen. Proliferation of fibroblastic forms was not entirely inhibited, however, for some of the isolated cells formed small colonies before they degenerated and disintegrated; but in general the macrophages and fibroblastic forms developing from the mononuclear cells derived from the tuberculous animals were much more inhibited in the presence of old tuberculin than were cells from normal animals.

Sensitivity of Splenic Explants to Tuberculin.—Splenic explants from normal and tuberculous animals were tested with old tuberculin in a concentration of 1 to 300. The splenic cells from the tuberculous animals were markedly sensitive to tuberculin. This confirmed results obtained previously (1).

Persistence of Cellular Sensitivity to Tuberculin of Fibroblasts Derived from Mononuclear Cells.—Since explanted mononuclear exudative cells derived from tuberculous animals exhibited sensitivity to the toxic effect of tuberculin similar to that exhibited by splenic cells, it seemed desirable to determine also whether this cellular sensitivity persisted on transplantation of fibroblasts derived from mononuclear cells. Such transplantation experiments were performed in Experiment 247, in which the cells were obtained from a guinea pig infected with strain R 1 tubercle bacillus for 1 month. One colony of fibroblasts which developed from a small clump of mononuclear exudative cells derived from the tuberculous animal was excised after growing in normal media for 8 days; it was divided into two nearly equal sized transplants. A larger colony of fibroblasts derived from a clump of normal mononuclear cells was excised on the same day and divided into four nearly equal sized transplants. One of the transplants derived from cells of the tuberculous animal and two normal transplants were transferred to a flask containing human old tuberculin in a concentration of 1 to 300. Similar transplants were transferred to a flask containing normal media. With this experimental set up the environmental conditions were controlled as completely as possible, since the two kinds of transplants were grown in identical media and in the same flasks.

The fibroblasts from mononuclear cells originally obtained from the tuberculous animal, grew as well in the flask containing normal media, as did the normal fibroblasts. Distinct differences were noted, however, in the fibroblastic growths in the flask containing tuberculin. The normal fibroblasts were only slightly affected as evidenced by slightly increased granulation of the cytoplasm. The cells originally derived from the tuberculous animal were markedly affected. They became very dark in color, heavily granulated, and swollen. Quantitatively there was a distinct inhibition of fibroblastic growth from the sensitive transplant in media containing tuberculin; while the fibroblastic growth from the normal transplants in tuberculin was not inhibited. The comparative cytotoxic index of tuberculin for sensitive cells on the 6th day after transplantation, or on the 14th day after the original explantation, was 0.51. The microscopic appearances of the two types of cells in the two different media are shown in Figs. 1 to 4.

For the second transplantation, the fibroblasts grown in normal media were excised and divided into two equal portions. One-half of each of the growths was transferred to a flask containing old tuberculin in a concentration of 1 to 300 and the other half was transferred to a flask containing normal media. Thus, a set up similar to the primary transplantation was provided. The sensitive fibroblasts were again specifically inhibited by tuberculin. The microscopic appearances of the two cell types in the two media 5 days after secondary transplantation are depicted in Figs. 5 to 8. The comparative cytotoxic index on the 6th day after the second transplantation, or 22 days after the original explantation, was 0.33.

A third transplantation was carried out as outlined for the secondary transplantation. Results similar to the two preceding transplantations were obtained. Fibroblasts derived from the original sensitive cells still displayed sensitivity to tuberculin both by microscopic cellular changes and by quantitative inhibition of fibroblastic growth. The comparative cytotoxic index on the 6th day after the third transplantation or, in other words, on the 29th day after original explantation was 0.42. The initial growth energy of the fibroblasts decreased during the last period so that further transplantations were not attempted.

DISCUSSION

Suspensions of scattered mononuclear cells were obtained from tuberculous animals by the intrapleural injection of warm molten parowax. This procedure incited the production of very little fluid exudate, for when the pleural cavity was exposed 5 to 7 days later, only a trace of fluid was observed. Such cells obtained practically free of body fluids by irrigation of the pleural cavity with Tyrode's solution exhibited marked sensitivity to the toxic action of tuberculin when tested in tissue culture.

These experiments offer, therefore, convincing evidence that sensitivity to tuberculin is an inherent characteristic of cells from tuberculous animals. This conclusion was also reached by Rich and Lewis (6) in tissue culture studies of tuberculin allergy, in which buffy coat and splenic explants were tested. The "summation effect hypothesis" seems untenable; this hypothesis predicates a combined toxic action of tissue fluids and tuberculin. In these experiments, however, the hypothetical tissue fluid toxin would appear to have been too dilute to exert any demonstrable toxic effect.

The transformation of mononuclear cells into sheets of fibroblasts was again demonstrated. Microscopic clumps or aggregates of mononuclear cells developed into macroscopic colonies of fibroblasts which were transplanted and were tested regarding their sensitivity to the toxic action of tuberculin. This inherent cellular characteristic persisted after prolonged culture *in vitro* and was still demonstrable after three transplantations, and over a period of 29 days. A similar persistence of cellular sensitivity to tuberculin *in vitro* was demonstrated on prolonged culture of splenic fibroblasts from tuberculous animals (1).

The nature of the toxic action of tuberculin on cells from tuberculous animals when tested *in vitro* is still a matter of conjecture. Rich and Lewis (6), reasoning by analogy with other types of hypersensitive states, in which antigen-antibody reactions are involved, are of the opinion that allergy in tuberculosis is also of the nature of an antigen-antibody union in which the antibody is bound to the cell.

Aronson (7) has shown that an antigen such as horse serum has no specific toxic effect when added to tissue cultures from animals sensitized to that foreign protein. We (8) obtained similar negative results when the respective antigens were tested on tissue cultures from

animals sensitized to horse serum, egg albumin, or beef lens. It is evident that bacterial proteins or other chemical fractions behave differently from the coagulable proteins when they are added to cultures of cells obtained from the respective sensitive animals.

Whether cells grown for a prolonged period *in vitro*, by repeated transplantations in the absence of the specific antigen, will continue to elaborate hypothetical sensitizing antibodies cannot be answered at present. Such a continued antibody formation would be necessary to explain the demonstrated persistence *in vitro* of cellular sensitivity to tuberculin, if this is due to an antigen-antibody union.

As yet, there is no conclusive proof that tuberculin allergy is the result of an antigen-antibody reaction, and these experiments fail to throw further light on this problem. Other tissue culture studies on hemolytic streptococcal allergy indicate that immune plasma has a neutralizing effect on the toxic action of streptococcal extract when tested on sensitive cells (9). The mixture of immune plasma, containing precipitins and agglutinins, with antigen apparently did not result in a product toxic for the cells. The question as to whether the union of antigen with a hypothetical antibody bound to a sensitive cell may exert a toxic effect on the cell, must await further experimental attack.

CONCLUSIONS

1. Mononuclear exudative cells, obtained from tuberculous guinea pigs by the intrapleural injection of parowax, exhibited characteristic sensitivity to the toxic action of tuberculin when tested in tissue culture.
2. Experiments with these cells, practically free of body fluids, show conclusively that sensitivity to tuberculin is an inherent characteristic of mesenchymal cells from tuberculous animals.
3. Fibroblastic growths which developed from mononuclear exudative cells derived from a tuberculous animal showed persistence of sensitivity to the toxic action of tuberculin on repeated transplantations over a prolonged period *in vitro*.

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EXPLANATION OF PLATES

PLATE 49

FIG. 1. Photomicrograph showing the effect of human old tuberculin 1 to 300 on first transplantation fibroblastic growth derived from a small clump of mononuclear exudative cells originally obtained from a guinea pig infected with strain R 1 tubercle bacilli for 1 month. 6 days after transplantation of fibroblasts or 14 days after original explantation of mononuclear exudative cells. Note the dark, swollen, heavily granulated cells. The dark area at the bottom is the central portion of the transplant, thus showing that cellular proliferation is distinctly inhibited. $\times 120$.

FIG. 2. Fibroblastic growth from control half of transplant similar to that in Fig. 1, but growing in normal media without tuberculin. The cells are healthy in appearance and growing actively. 6 days after transplantation. $\times 120$.

FIG. 3. Effect of human old tuberculin 1 to 300 on first transplantation fibroblastic growth originally derived from normal mononuclear exudative cells. 6 days after transplantation. The cells are practically unaffected by the concentration of tuberculin used. Compare with Fig. 1. The transplants in Fig. 1 and Fig. 3 were both grown in the same media and in the same flask. $\times 120$.

FIG. 4. Fibroblastic growth from control half of transplant similar to that in Fig. 3, but growing in normal media without tuberculin showing normal actively growing cells. The transplants in Fig. 2 and Fig. 4 were grown in the same flask. $\times 120$.

PLATE 50

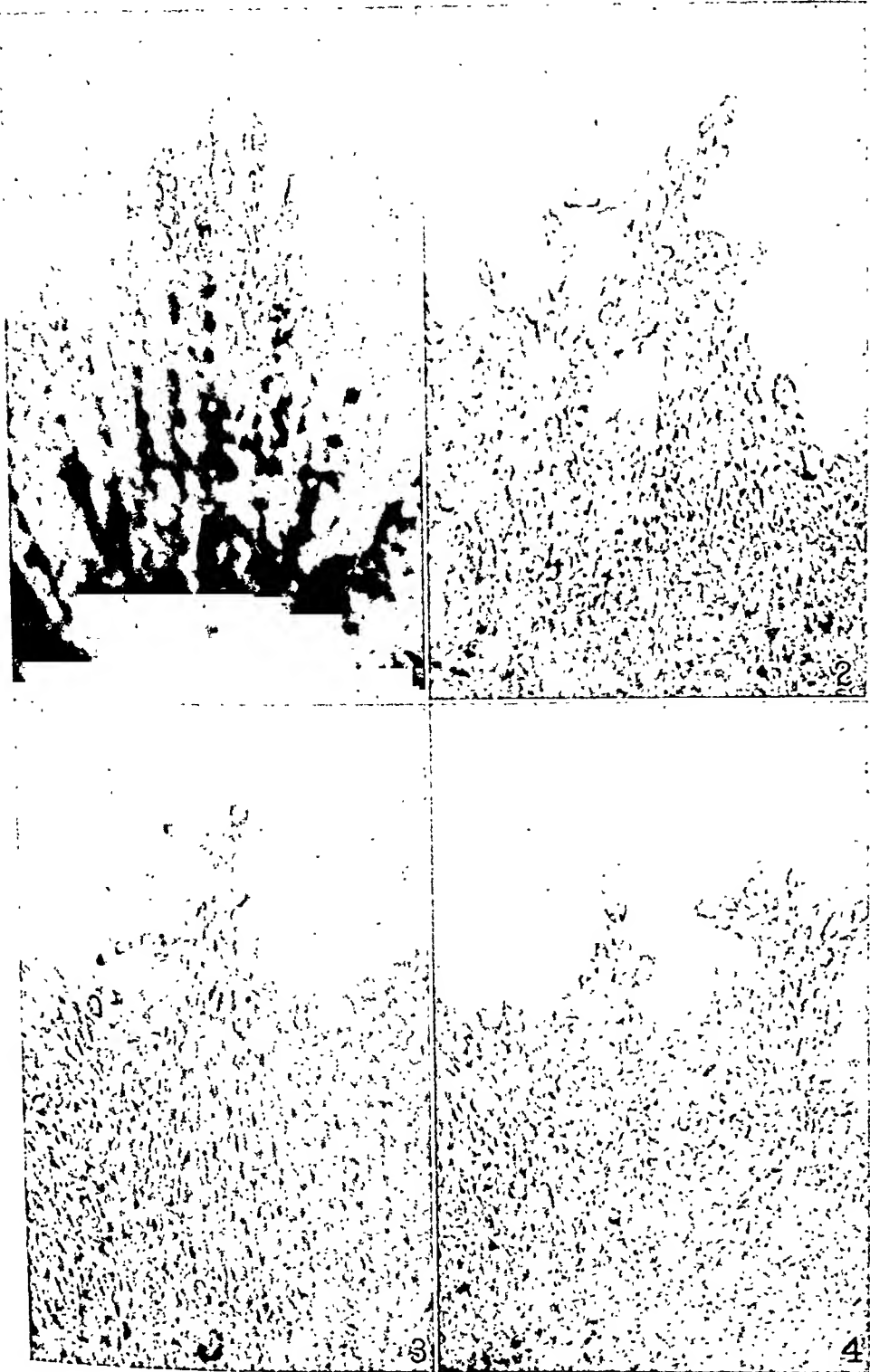
FIG. 5. Effect of tuberculin 1 to 300 on fibroblastic growth from secondary transplants originally derived from mononuclear exudative cells of guinea pig infected with strain R 1 tubercle bacillus. Transplant was obtained from fibroblastic growth shown in Fig. 2. 5 days after secondary transplantation or 21 days after original explantation. The toxic effect of tuberculin on the sensitive cells is again shown by the dark, swollen, heavily granulated cells and by inhibition of cellular proliferation. $\times 120$.

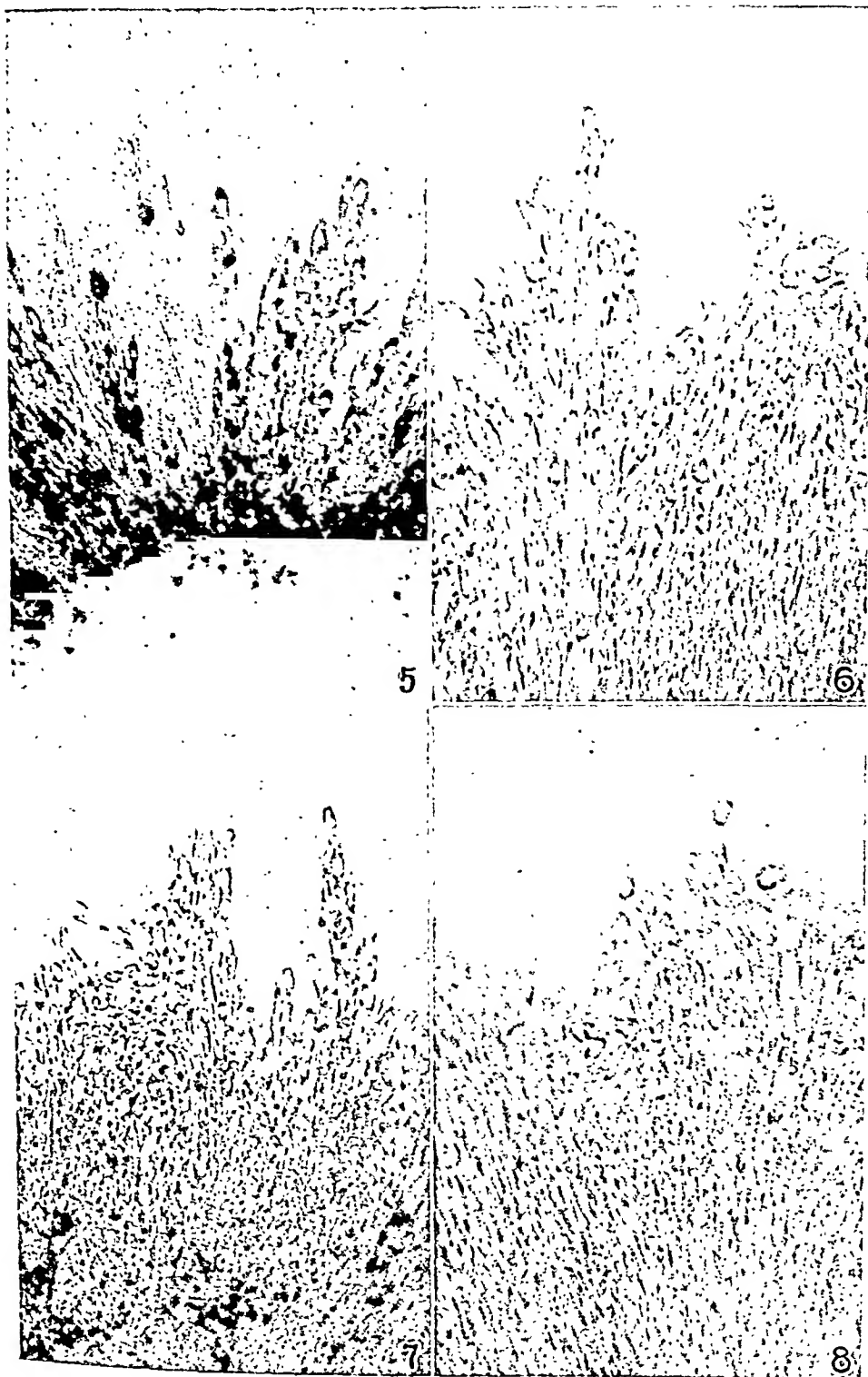
FIG. 6. Fibroblastic growth from control half of transplant similar to that in Fig. 5, which was obtained from fibroblastic growth shown in Fig. 2 and growing in normal media without tuberculin. The cells appear healthy and continue to grow actively. 5 days after secondary transplantation. $\times 120$.

FIG. 7. Effect of tuberculin 1 to 300 on fibroblastic growth from secondary

transplants originally derived from normal mononuclear exudative cells. Transplant was obtained from fibroblastic growth shown in Fig. 4. The cells are but slightly affected by this concentration of tuberculin. 5 days after secondary transplantation. Compare with Fig. 5. The transplants in Figs. 5 and 7 were grown in the same flask. $\times 120$.

FIG. 8. Fibroblastic growth from control half of transplant similar to that in Fig. 7 but growing in normal media and showing normal actively growing cells. 5 days after secondary transplantation. The transplants shown in Figs. 6 and 8 were grown in the same flask. $\times 120$.





EFFECT OF PNEUMOCOCCUS AUTOLYSATE ANTI-TOXIN ON PNEUMOCOCCUS PNEUMONIA IN GUINEA PIGS

By JULIA T. WELD AND HELENA GILDER

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York)

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In previous papers (1-3) we showed that the Berkefeld filtrates of certain anaerobically produced autolysates of pneumococcus of Types I, II, or III cause in guinea pigs: (a) necrosis of the skin when injected intracutaneously; (b) hemorrhagic edema of the lungs and death when injected intratracheally; (c) pneumonia associated with unrestrained multiplication of the organism in the lung when injected intratracheally in sublethal doses combined with small amounts of living organisms; and cause in mice death with kidney lesions, when injected intravenously. We also demonstrated that immunization of horses with these autolysates stimulates the production of sera of marked antitoxic properties for the principles that cause, in guinea pigs necrosis of the skin and hemorrhagic edema of the lungs, and in mice kidney lesions and death (4, 5). Moreover, an antiautolysate serum, prepared by the immunization with the autolysate from one type of pneumococcus, neutralized the poisons affecting the skin and the lung produced from each of the other 3 types, demonstrating that these autolysate antitoxins and presumably the toxins are not type specific (1, 4, 5).

Despite much work toward the understanding of the pathogenesis of pneumococcus pneumonia, there are many obscure features of the disease that have never been explained satisfactorily. If substances of the nature of the toxic autolysates could be demonstrated *in vivo* in animals having pneumococcus pneumonia, such a finding would help explain the phenomenon of lobar pneumonia in man. It was with the object of determining whether poisons similar to those in

the toxic autolysates are produced *in vivo*, that this work was undertaken.

If the autolysate toxin is produced *in vivo* during the growth of the organism in the tissues, one would expect that the autolysate antitoxin, through neutralization of the toxin, would at least delay death in animals injected with the pneumococcus. This, however, is not the case. We found that autolysate antitoxin injected even in large amounts is unable to protect or prolong the life of guinea pigs or mice injected intramuscularly or intraperitoneally with a lethal dose of pneumococcus. Moreover, Sabin (6) showed that our autolysate antitoxin, when used by itself or in combination with a so called sub-effective dose of specific antibacterial serum, had no apparent effect on the progress of pneumococcus infection in mice injected intraperitoneally or in rabbits injected intracutaneously with large doses of virulent pneumococci. It seems, therefore, evident from our own work and from that of Sabin that toxic substances of the nature of those in the toxic autolysates neutralizable by the autolysate antitoxin, are not produced in the animal body during generalized infection with the pneumococcus.

However, there appears to be another way of looking at this problem. In pneumococcus pneumonia in man and in the guinea pig, pneumonia occurs with growth of the organisms in the lung, frequently without invasion of, or establishment of, the pneumococcus in other organs or tissues. Is it possible, then, that the poisons in the autolysates, especially the toxin that is injurious to the lungs, enabling pneumococci to grow in them, are produced *in vivo* only in the lung? If this is the case, the autolysate antitoxin, through neutralization of these poisons in the lung, might have a curative effect in pneumonia in delaying or arresting the spread of pneumococcus infection. Indirectly this neutralizing action in the lung might prevent the occurrence of a generalized infection, although it would have no direct effect on a pneumococcus sepsis once established. In an endeavor to answer this question we have produced pneumonia in guinea pigs and attempted to protect them with autolysate antitoxin.

Experimental Methods

Production of Pneumonia in Guinea Pigs.—Pneumonia was produced in guinea pigs by the three following methods.

1. Intratracheal injection of large amounts of living virulent pneumococci. (Pneumococci from 3 to 4 cc. of culture in 0.2 cc. volume.)

These pigs usually get moderately extensive pneumonia lasting about 2 days. In 2 instances a pig in this group died with an extensive pneumonia associated with unrestrained growth of pneumococci in the lung, similar to the pneumonia caused by the intratracheal injection of a mixture of small amounts of autolysate toxin and living pneumococci. All animals in this group died with a pneumococcus sepsis 2 to 5 days after injection.

2. Intratracheal injection of large amounts of living virulent pneumococci into guinea pigs prepared either immediately previously or 18 hours previously by intraperitoneal injection of small amounts of homologous antibacterial (horse) sera (7, 8). (Pneumococci from 4 to 6 cc. of culture in 0.2 cc. to 0.25 cc. volume.)¹

These pigs usually develop extensive pneumonia and die 1 to 4 days after injection with sepsis. (The lung involvement is greater than that produced by method 1.) Occasionally the guinea pigs of this group develop extensive pneumonia associated with unrestrained growth of the organism in the lung. Very rarely an animal of this group survives.

3. Intratracheal injection of a small amount of living pneumococcus organisms combined with sublethal doses of staphylococcus toxin.

These pigs usually develop extensive pneumonia in 1 to 4 days after injection and sepsis. Frequently the pigs in this group develop extensive pneumonia associated with unrestrained growth of the organisms in the lung.

Pneumococcus Cultures Used.—A *Pneumococcus* Type I and a *Pneumococcus* Type II strain were used for inoculation of the guinea pigs in this work in all 3 groups of guinea pigs. Both strains were obtained from the blood of patients with lobar pneumonia at the Presbyterian Hospital. The virulence of the strains was kept up by passage through guinea pigs. The organisms were grown under aseptic conditions on a double strength veal infusion broth containing 4 per cent Witte peptone. After 12 to 14 hours growth, the pneumococcus culture was iced and centrifuged and the organisms taken up in the required amount of the supernatant broth. The pneumococcus suspension was then adjusted to a pH of 7 to 7.2 and kept in ice water during the experiment.

Staphylococcus Toxin.—The staphylococcus toxin used in this work was prepared from a hemolytic *Staphylococcus aureus* strain recovered from the blood of a case of staphylococcus septicemia in October, 1934. When injected intratracheally in guinea pigs in 0.1 cc. amounts, it produces hemorrhagic edema of the lungs and death.

Sera Used.—Pneumococcus autolysate antitoxin: The autolysate antitoxin used in these experiments was a concentrated and refined serum obtained from horses immunized² to the sterile toxic autolysate filtrates from *Pneumococcus* Types I, II, and III. This antitoxin contains no specific antibacterial substances for

¹ In this work we shall speak of these partly immune guinea pigs as sensitized.

² By Eli Lilly and Co. in 1928 and 1929.

Pneumococcus Types I, II, or III. At present it has a titre of 4500 guinea pig units and 7800 mouse units per cc.

Specific antibacterial sera: Antibacterial serum Type I—New York Board of Health. Refined—containing 2500 units per cc. Antibacterial serum Type II—New York Board of Health. Refined—containing 3000 units per cc.

Normal horse serum: Inactivated.

*Effect of Pneumococcus Autolysate Antitoxin on the Pneumonia
Caused by the Intratracheal Injection of Large
Amounts of Pneumococcus*

In Table I is presented a protocol of an experiment in which autolysate antitoxin or normal horse serum was given intraperitoneally immediately after the intratracheal inoculation of pneumococcus suspension, and demonstrates clearly the protective effect of the antitoxin. There is a discrepancy in this experiment in that guinea pig 10 that received antitoxin died in 1 day. In a large number of animals inoculated intratracheally with large amounts of living pneumococci, we have found that there is a small percentage of guinea pigs (approximately 10 per cent) that appear to be especially susceptible to the organisms and we believe that a much greater susceptibility than normal explains the early death of this guinea pig. An instance of this in another experiment was the death of a guinea pig in 2 hours after the intratracheal inoculation of pneumococcus. This guinea pig showed at autopsy lungs already large, with patchy hemorrhagic pneumonia throughout. 4 guinea pigs inoculated with the same quantity of organisms as this latter guinea pig survived for 2 or 3 days. In respect to guinea pig 10, Table I, it is of interest to note that the culture from its heart blood was negative, whereas in all the 8 control guinea pigs, positive heart blood cultures were obtained. This occurred in another experiment in which 1 of 9 guinea pigs inoculated with antitoxin died and it had a negative heart culture at autopsy, while all the controls died having positive heart cultures.

Several experiments similar to the experiment, Table I, with minor variations have been carried out, always with substantially the same results. We found that one-half the amount of antitoxin injected in the experiment, Table I, *viz.*, 1 cc. of 1-2 dilution, was sufficient to save the animals. As shown in experiment, Table I, and confirmed many times in other experiments, normal inactivated horse serum

TABLE I
Effect of *Pneumococcus Autolysate Antitoxin* on the Pneumonia Caused by the
Intratracheal Injection in Guinea Pigs of Large
Amounts of *Pneumococci*

Guinea pig No.	Weight gm.	<i>Pneumococcus</i> suspen- sion intratracheally	Serum intraperitoneally 2 cc. 1-2	Symptoms	Died or survived	Amount of consolida- tion in lungs	Culture heart	Culture lungs	Remarks
1	194	0.2	—	+++	D 2 days	±	+	++	
2	199	"	—	++	D 3 "	++	+	++	
3	210	"	—	+++	D 2 "	+++	+++	+++	
4	215	"	—	+++	D 3 "	+++	+	+	
5	193	"	Normal horse	++	D 4 "	+	+	+++	
6	198	"	" "	+++	D 2 "	+	+	+	
7	207	"	" "	+++	D 1 day	++	++	++	
8	211	"	" "	++	D 2 days	+++	+	+	
9	189	"	Autolysate anti-toxin	0	S	0	0	++	Killed 5 days after intratracheal inoculation
10	195	"	" "	+++	D 1 day	++	0	++	
11	200	"	" "	0	S	0	0	±*	Killed 5 days after intratracheal inoculation
12	210	"	" "	0	S	0	0	±*	" "

Pneumococcus I Suspension.—The organisms from 3 cc. of culture were inoculated into each guinea pig. The normal horse serum or antitoxin was inoculated intraperitoneally into each pig immediately after the intratracheal inoculation of organisms. One animal from each group (no serum, horse serum, antitoxin) was inoculated, then a second animal from each group and so forth, so that any change that might possibly occur in the suspension of pneumococci during the time taken to inoculate would be distributed equally among the 3 groups of guinea pigs. This procedure was carried out in all our other experiments.

* In the lung culture column, ± indicates less than 8 colonies on plate.

TABLE II

The Effect of Antiautolysate Serum on the Pneumonia Caused in Sensitized Guinea Pigs by the Intratracheal Inoculation of Large Doses of Living Pneumococci

Guinea pig No.	Weight	Feb. 17	Feb. 18	Feb. 18 Serum intraperitoneally 1-10 dilution	Feb. 18 Pneumococcus suspension intratracheally cc.	Symptoms	Died or survived	Amount of consolidation in lungs	Culture heart	Culture lungs	Remarks
		Serum intraperitoneally 1 cc. 1-2 dilution	Serum intraperitoneally 1 cc. 1-2 dilution								
1	207	—	—	Antipneumococcus I, 0.5 cc.	0.25	+++	D 3 days	+++	++	+++	
2	201	—	—	"	"	+++	D 4	++	0	0	
3	195	—	—	"	"	+	S	±	0	0	4 days after intratracheal inoculation well. Killed
4	200	Normal horse	Normal horse	"	"	+++	D 2 days	+	+	+++	
5	194	"	"	"	"	++	D 4	+	+	+	
6	193	"	"	"	"	+++	D 2	+++	++	+++	
7	204	Autolysate antitoxin	Autolysate antitoxin	"	"	+	S	0	0	0	4 days after intratracheal inoculation well. Killed
8	194	"	"	"	"	0	S	±	0	0	4 days after inoculation, well. Killed
9	198	"	"	"	"	+	S	0	0	0	4 days after inoculation, well. Killed

Pneumococcus I Suspension.—Each guinea pig was inoculated with the organisms from 4.5 cc. of culture. The first inoculation of normal horse serum or antiautolysate serum was given 20 hours, the second inoculation of normal horse serum or antiautolysate serum 2 hours before the intratracheal inoculation of pneumococci. The intraperitoneal inoculation of antibacterial serum was given immediately preceding the intratracheal inoculations.

has no beneficial effect against the intratracheal inoculation of pneumococci. There appears to be no doubt, therefore, that the autolysate antitoxin under the conditions of these experiments saves the great majority of animals injected intratracheally with large doses of living pneumococcus.

Effect of Autolysate Antitoxin on the Pneumonia Caused in Specifically Sensitized Guinea Pigs by the Intratracheal Inoculation of Large Doses of Living Pneumococci

In Table II is presented a protocol of an experiment in which autolysate antitoxin or normal horse serum was given intraperitoneally 1 day and 2 hours before the intratracheal inoculation of pneumococci into sensitized guinea pigs. It is seen that only one of the 6 control guinea pigs survived whereas all the antitoxin treated guinea pigs survived, demonstrating the efficacy of the antitoxin against the pneumonia under the conditions of this experiment.

This type of experiment (Table II) has been repeated several times with variations as to the time at which the antitoxin and specific antibacterial serum were given in respect to the intratracheal inoculation of organisms, always with essentially the same results. In one experiment the autolysate antitoxin was given intravenously 18 hours after the injection of pneumococcus into sensitized guinea pigs at a time when the guinea pigs had symptoms of well advanced pneumonia, and the antitoxin treated guinea pigs lived much longer than the control pigs, or survived.

These experiments, therefore, indicate that the autolysate antitoxin in most instances saves guinea pigs from an almost surely fatal pneumonia caused by the injection of large doses of pneumococcus into specifically sensitized guinea pigs.

Effect of Autolysate Antitoxin on the Pneumonia Caused by the Intratracheal Injection of Mixtures of Staphylococcus Toxin and Pneumococcus Culture

Although, as has been pointed out previously, pneumococcus autolysate antitoxin has no effect on the progress of a systemic infection with the pneumococcus, from the foregoing experiments it is evident that the injection of autolysate antitoxin saved the great majority of

guinea pigs from an almost surely fatal pneumonia. This would seem to favor the conjecture that the toxin or toxins in pneumococcus pneumonia are elaborated in the lung. But there appears to be another possible explanation of these results. The suspension of pneumococci injected to produce pneumonia contained per cubic centimeter approximately only 1/30 of the organisms present in the suspension from which the autolysate toxin was produced. However, it seems conceivable that in the lung and bronchi enough of the introduced pneumococci could have been autolyzed so as to produce sufficient toxin to cause a primary injury, thus enabling the pneumococci to become established in the lung.

If this were the case, the beneficial effect of the autolysate antitoxin could be ascribed to the neutralization of the poison or poisons assumed to have been produced from the *in vivo* autolysis of the *inoculated* pneumococci rather than to the neutralization of the poisons produced during the growth and autolysis of the *freshly growing* pneumococci in the lung. In such case the antitoxin might have prevented the start of the infection and saved the animal. From this reasoning, therefore, it appears that the foregoing experiments furnish no proof of the production of the autolysate poison *in vivo* from organisms growing in the lung.

We have already shown that autolysate antitoxin prevents the development of pneumonia and death otherwise induced by the intratracheal injection of small doses of living pneumococci combined with sublethal doses of pneumococcus autolysate poison (5). But here again the beneficial effect of the antitoxin might be ascribed to the neutralization of the inoculated toxin and not to the neutralization of the autolysate toxin possibly elaborated in the lung by the growth of the organisms there.

It seemed to us that one way of answering this question was to infect animals intratracheally with doses of pneumococci too small to cause pneumonia by themselves, combined with a substance toxic for the lung but nonspecific for the pneumococcus, for the purpose of enabling the organisms to become established in the lung and thus cause pneumonia. If antitoxin were found effective in this kind of pneumonia, it would be logical to infer that the antitoxin had neutralized poisons produced in some way by the growing and autolyzing

organisms and that therefore the autolysate poison or poisons are presumably produced in the lung. With this idea in mind, we produced pneumonia in guinea pigs by injecting them intratracheally with sublethal doses of staphylococcus toxin, which is toxic for lungs

TABLE III
Effect of Pneumococcus Autolysate Antitoxin on the Pneumonia Caused in Guinea Pigs by the Intratracheal Injection of Mixtures of Pneumococcus Culture and Staphylococcus Toxin

Guinea pig No.	Weight gm.	Pneumococcus antitoxin	Pneumococcus culture + staphylococcus toxin intratracheally	Symptoms	Died or survived	Amount of consolidation in lungs	Culture heart	Culture lungs	Remarks
1	205	—	0.2	+++	D in less than 18 hrs.	+++	+++	+++	
2	175	—	"	++	S	0	0	0	6 days after intra- tracheal injection, well. Killed
3	172	—	"	+++	D in less than 18 hrs.	++	0	+	
4	192	2 cc. 1-2	"	+	S	0	0	0	6 days after intra- tracheal injection, well. Killed
5	181	" " "	"	+	S	0	0	0	" "
6	172	" " "	"	+	S	0	0	0	" "

The pneumococcus antitoxin, diluted 1-2, was injected intraperitoneally 4 hours before the intratracheal inoculation. 0.3 cc. of *Pneumococcus* Type I (14 hours growth) was mixed with 0.1 cc. of staphylococcus toxin and 0.2 cc. of the mixture inoculated intratracheally into each guinea pig.

when injected intratracheally, mixed with small doses of living pneumococci and were able to save most of these animals with injections of autolysate antitoxin previously administered. Table III gives the results of one of the experiments of this kind. This experiment which has been repeated several times always with

substantially the same results demonstrates that pneumococcus autolysate antitoxin has a definitely beneficial effect on the pneumonia caused by the injection of mixtures of staphylococcus toxin and the pneumococcus.

Regarding further control experiments, which are not included in the tables, it has been found: (a) that the pneumococcus antitoxin has no more detoxifying effect on the staphylococcus toxin (when mixed *in vitro* and inoculated into guinea pigs) than has normal horse serum, and that, therefore, the results of the above experiments are not caused by a nonspecific neutralization or destruction of the staphylococcus toxin; and (b) that normal horse serum given intraperitoneally has no effect in prolonging the life or saving the guinea pigs inoculated with pneumococcus culture + staphylococcus toxin mixtures.

These experiments with staphylococcus toxin show clearly that the antitoxin neutralizes the poison or poisons produced in the lung by the invading and growing organism and that presumably these poisons are the same as those present in the toxic autolysate.

DISCUSSION

The experiments in this paper demonstrate that pneumococcus autolysate antitoxin under certain conditions is efficacious against pneumococcus pneumonia in guinea pigs. The plausible explanation of these results is that the antitoxin neutralizes poisons manufactured by the growing and autolyzing pneumococci in the lung, thus preventing the spread of the infection by preventing damage to the tissues. Since the antitoxin is produced by the immunization of horses with the toxic autolysates and since it neutralizes *in vitro* all the so far determined poisons contained in the autolysates, and *in vivo* the toxin that enables pneumococci to grow in the lung,³ it is reasonable to believe that the poisons neutralized by the antitoxin in the lung are similar to those present in the toxic autolysates.

Whether all the toxic effects of the autolysates are important in the production of pneumonia is impossible to say. The toxins that enable pneumococci to grow in the lung would appear to be the most important toxins in this respect. That the dermatotoxic substance in the autolysates has little if any importance in pneumococcus infec-

³ Weld, J. T., unpublished experiments.

tions appears probable from the work of Sabin. He showed that the autolysate antitoxin had no beneficial effect against the Goodner (9) so called intradermal pneumonia in rabbits caused by the intradermal infection by virulent pneumococci. Even large doses of autolysate antitoxin did not appear to delay or arrest the development of the local lesion nor to modify the fully developed lesion.

If it is confirmed that the pneumococcus autolysate toxin is produced only in the lung in pneumonia, it would seem that in pneumococcus infections one must distinguish between poisons that are produced in the lung and those that are produced when the organism is growing in other tissues generally. The dermatotoxic and fever producing toxins of Coca (10) may possibly belong in the latter class of poisons.

CONCLUSIONS

1. In rabbits, guinea pigs, and mice pneumococcus autolysate antitoxin has no effect against generalized infection by the pneumococcus.
2. In a large proportion of cases of pneumonia and sepsis caused by the intratracheal inoculation of large amounts of living pneumococci in normal or sensitized guinea pigs, autolysate antitoxin saves the animal.
3. The autolysate antitoxin is protective in most cases of pneumonia and sepsis caused by the intratracheal inoculation of small amounts of living pneumococci combined with sublethal doses of staphylococcus toxin.
4. These experiments indicate that in pneumococcus pneumonia of guinea pigs the autolysate toxin or toxins are elaborated only in the lungs.

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THE EFFECT OF EXPERIMENTAL REDUCTION OF KIDNEY SUBSTANCE UPON THE PARATHYROID GLANDS AND SKELETAL TISSUE

By ALWIN M. PAPPENHEIMER, M.D.

WITH THE ASSISTANCE OF ANNA HART

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York)

PLATES 51 TO 54

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There are many established facts pointing to a relationship between kidneys and parathyroids.

1. MacCallum (1), Bergstrand (2), Hubbard and Wentworth (3), Pollack and Siegal (4), Magnus and Scott (5), have demonstrated the occurrence of parathyroid hyperplasia in individual cases of chronic nephritis. Pappenheimer and Wilens (6) have shown that such enlargement occurs regularly, and is proportional to the degree of renal damage.

2. Renal lesions occur in approximately 50 per cent of the cases of hyperparathyroidism with osteitis fibrosa (Albright, Baird, Cope and Bloomberg (7)). These are associated with formation of calculi (Type I) or with a diffuse calcinosis (Type II). The calcium content of the kidneys may be greatly elevated by injection of parathormone in suitable doses (Molinari-Tosatti (8), Olsen (9), Spingarn (10)).

3. In the few cases of renal rickets in which the parathyroids have been studied, they have been found to be enlarged (Langmead and Orr (11), Smyth and Goldman (12), Schelling and Remsen (13)). The last authors, using the technique of Hamilton and Schwartz (14), were able to demonstrate an increased amount of parathyroid hormone in the blood during life.

4. Indirect evidence of a relationship between parathyroid and renal function is seen in the facts that (a) injection of parathormone is followed almost immediately by an increased urinary excretion of

phosphate (Greenwald and Gross (15), Albright and Ellsworth (16), Albright, Bauer, Roper and Aub (17)); (b) that this does not occur in nephritics in whom the excreting power of the kidneys is impaired (Goadby and Stacey (18)); (c) that after complete ablation of kidneys in dogs, injection of parathormone fails to cause a significant rise of calcium in the blood above that of untreated nephrectomized controls. The mobilization of the calcium store of the body by parathyroid hormone thus depends on kidney function (Tweedy, Templeton, McJunkin (19)); (d) that there is increase in the phosphatase activity of the kidney after parathyroidectomy, and decrease after parathormone injections (Pisa (20)). We shall not attempt to discuss the theoretical implications of these several observations. They have been cited to show that there is considerable evidence of an interrelation between parathyroids and kidneys, and need of further experimentation on the subject.

The concrete questions which we shall attempt to answer in this paper are these:

1. Does experimental reduction of kidney tissue lead to significant hyperplasia of the parathyroid glands?
2. If so, is this increased functional activity reflected in alterations of the osseous tissue, and is it possible to produce experimentally in rats a picture analogous to renal rickets in man?

EXPERIMENTAL

In the first series of experiments,¹ healthy white rats weighing from 150 to 250 gm. were used. They were kept on a mixed diet.² One kidney was removed through a lumbar incision, and a considerable part of the opposite kidney was destroyed by thermocautery in two subsequent operations.³

The animals were allowed to live for varying periods of time; when they were killed or died spontaneously, a litter mate unoperated control was killed for comparison.

The volume of the parathyroids was determined in the following way. The glands, with attached thyroid tissue, were fixed in Zenker's fluid, and serially

¹ For preliminary report of these experiments, see Jarrett, W. A., Peters, H. L. and Pappenheimer, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1935, 32, 1211.

² A commercial chicken mash, containing cereals, alfalfa, meal, meat scraps, skimmed milk, cod liver meal, sardine oil, etc. Rats on this diet show excellent growth and fertility.

³ All operations were performed under ether anesthesia.

sectioned at 10μ . Their volume was calculated by multiplying the combined areas of the sections, as obtained with the planimeter from drawings projected at known magnification, by the thickness of the section. The volume was calculated for 100 gm. of weight. Since many of the partially nephrectomized rats lost weight before death, the calculations were based upon the normal weight at time of death, as estimated from Donaldson's tables, and not upon the actual weight.

In addition to the parathyroids, sections were prepared from kidneys and bones, the latter decalcified by prolonged fixation in Mueller's fluid. After a preliminary period of apparent health, the majority of the rats after the second cauterization showed a stationary or declining weight curve, drowsiness, roughness of the hair and loss of appetite.

The operated rats fall into two groups according to the time of survival. Those allowed to live for a period of 113 to 124 days after the second cauterization showed much more intense lesions of the remaining kidney tissue than did those in the group killed or dying before 46 days. The data are summarized in Table I.

TABLE I
Average Combined Volume of Parathyroids per 100 Gm. of Rat

	Volume	PE_m	Standard deviation
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
Group A—Controls (9).....	0.1441	0.0130	0.0551
Group B*—Early nephritics (5).....	0.1679	0.0066	0.0197
Group C*—Late nephritics (5).....	0.4117	0.0553	0.1659

* Calculations of B and C are based on estimated normal weights at time of death.

Comparing the combined volume of the parathyroids per 100 gm. of rat in the operated and control animals, we find, in spite of the small numbers of animals, a decided increase in parathyroid volume in the partially nephrectomized rats as compared with the controls. In the group which survived a longer period and showed more intense renal lesions, the difference is statistically valid; in the earlier group it is suggestive but not conclusive. although in each rat the volume of the gland exceeded the mean of the control series.

One may reasonably conclude—and this will be supported by subsequent experiment—that the reduction of functional renal tissue has led to a decided increase in the size of the parathyroids.

The question arises as to whether this increase is to be ascribed to enlargement of the cells, or to their multiplication. Measurements

of the nuclei in two diameters indicated that the nuclei in the glands of operated rats were larger than those of the controls. Thus the mean diameters of 250 nuclei in the 5 nephritic rats were $8.06 \times 6.2 \mu$ as against $6.8 \times 4.4 \mu$ in normal nuclei. It could also be demonstrated that the increase in nuclear size was attended by an increase in cytoplasmic volume. Mitotic figures were rarely found. It would seem that the increase in the total bulk of the gland may be accounted for in part, at least, by the increased volume of both nucleus and cytoplasm.

The pathologic changes which develop in the remaining kidney substance in the later group C, are both diffuse and severe, simulating an advanced stage of glomerulonephritis (Fig. 1). At least 90 per cent of the glomeruli are greatly enlarged, bloodless, the capillary loops distended with hyaline and granular material, the capsular space obliterated by adhesions (Fig. 2). Often there is a crescentic proliferation of epithelial cells. In many tufts tissue changes progress to complete obliteration.

The majority of the tubules are greatly dilated, and their lumina filled with dense hyaline coagulum. The epithelial cells are flattened, so that in some areas the tissue resembles thyroid. There is irregular interstitial fibrosis with moderate lymphoid infiltration of the stroma. In a few of the larger arteries and in some of the arterioles there is fibrinoid or hyaline material in the subendothelial tissues.

With minor variations in intensity, the same picture is found in all 5 animals of the later group. Although the pathogenesis of the lesions is not clear, it is of interest that the cauterization leads to the gradual development of a diffuse nephritis with glomerular lesions comparable to those of advanced human glomerulonephritis. Such diffuse changes were not seen in the kidneys of group B killed within 46 days of the last cauterization.

The second series of experiments is essentially a repetition of the first, save that many of the rats were operated upon at an earlier age, and kept under observation for longer periods. Excluding those that died prematurely or of incidental causes, there remained 16 operated, and 12 control rats. The additional data afforded by these experiments are summarized in Table II.

These figures amply confirm those of the earlier experiments. In spite of their lower body weight, there is an approximately 100 per cent increase in the mean parathyroid volume of the operated rats, over

that found in their litter mate controls. Calculated on the basis of maximal weight, and expressed as volume of parathyroid per 100 gm. of rat, the difference is still more striking. The severity of the renal lesions was graded without knowledge of the parathyroid measurements. It is seen that the degree of hypertrophy is closely correlated with the intensity of the kidney lesions. In one instance in which the nephritic changes were pronounced, the parathyroids were 5.6

TABLE II
Volume of Parathyroid Glands in Normal and Nephritic Rats

No.	Normal controls		Nephritic rats		Severity of kidney lesions
	Absolute volume	Volume per 100 gm. (maximum weight)	Absolute volume	Volume per 100 gm. (maximum weight)	
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	
1	0.536	0.158	0.675	0.262	++
2	0.235	0.066	0.462	0.165	+
3	0.418	0.167	1.31	0.612	++++
4	0.307	0.135	0.433	0.147	+
5	0.403	0.175	0.755	0.352	++
6	0.540	0.135	0.931	0.417	+++
7	0.518	0.173	0.686	0.214	++
8	0.180	0.090	0.709	0.296	++
9	0.541	0.183	1.52	0.709	++++
10	0.432	0.141	0.398	0.148	+
11	0.590	0.147	0.945	0.387	++++
12	0.400	0.167	0.528	0.272	++
13			0.362	0.179	±
14			0.800	0.252	±
15			2.421	0.792	+++
16			0.700	0.286	±
Mean.....	0.425	0.145	0.852	0.343	

times as large as those of the control litter mate of the same sex, age and weight. In every instance, regardless of the severity of the renal lesions, both absolute and relative volume in the operated rats exceeded the mean volume of the controls.

The question may be raised at this point as to a possible sex difference in parathyroid volume. In human beings, Pappenheimer and Wilens (6) found that the mean weight of the female glands during the

period of active sex life was 22 per cent in excess of the male glands. In the small series of 10 male and 11 female unoperated controls, the mean volume per 100 gm. of rat was 0.1610 c.mm. in the females as against 0.1348 c.mm. in the males—a preponderance of about 20 per cent. The significance of this sex difference and its possible relation to certain phases of the reproductive cycle is being studied by Peters and Andersen, and will be reported upon elsewhere. The point of interest in connection with the present problem is that the enlargement due to reduction of kidney substance greatly transcends that attributable to sex difference.

It was expected that the parathyroid hypertrophy might bring about skeletal changes in the direction of osteitis fibrosa. In the second group of 16 rats surviving from 148 to 306 days after the second operation, only 2 showed histological changes in the bones, and these were of a trifling character. The epiphyses were normal for the age. In rat B7 the perforating vessels of the cortex in femur and tibia were surrounded by fibrous tissue in which the fibers were impregnated with calcium. There was increased osteoclastic resorption. Rat H7 showed areas of marrow fibrosis and a few osteoclasts in the rib (Fig. 3). In the other cases in spite of the renal insufficiency and parathyroid enlargement, no evidence of excessive bone resorption was found.

In view of these negative results further experiments were performed which differed (a) in that the rats were operated upon at an earlier age, (b) the operative technique was modified in the hope of effecting a still greater reduction in functional kidney tissue, (c) the calcium intake was reduced to various levels.

The operation was a modification of that devised by Chanutin and Ferris (21) for the study of renal insufficiency in rats. The kidney exposed through a lumbar incision was delivered through the wound, and both poles snipped off with sharp scissors. The cut surfaces were then seared with a hot knife, no ligature being used. The remaining kidney tissue, reduced to about $\frac{1}{2}$ or $\frac{1}{3}$ of the original volume, was replaced and peritoneum and skin closed with sutures. A few days later the opposite kidney was removed *in toto*. The wounds invariably healed without infection.

The operations were performed when the rats were 10 to 15 days old. After weaning, they were placed on the following low calcium diet, with or without a supplement of CaCO_3 .

Basal Diet

	Per cent	Calcium	Mg. calcium in 100 gm.
Yellow corn.....	54.0	<i>per cent</i> 0.0245	0.0132
Whole wheat.....	16.5	0.0324	0.0053
Casein (commercial).....	17.5	0.26	0.0195
Yeast.....	10.0	0.0865	0.0086
		Total calcium....	0.0466 mg.

No antirachitic was added, and direct sunlight was excluded. The unoperated controls on this low calcium diet showed a fairly satisfactory but not entirely normal growth curve, whereas the partially nephrectomized animals lagged behind and in most instances were stunted in their development; after an initial period of growth the weight curve became stationary or declined. The dwarfed appearance of the operated animals in comparison to their litter mate controls is well shown in Fig. 4.

As was anticipated from the work of Luce (22), the lowered calcium content of the diet in itself led to a noteworthy increase in the volume of the parathyroids. Thus the mean volume per 100 gm. in 14 unoperated controls on low calcium diet killed at various ages was 0.305 c.mm., as compared with 0.144 and 0.145 in the two series given the stock laboratory diet with adequate calcium.

The hyperplasia of the parathyroid in the operated rats, however, was very much greater than in the controls. In spite of the retarded growth, the mean absolute volume in 8 operated rats was almost double that of the controls (0.657 c.mm. as compared with 0.376 c.mm.); the volume per 100 gm. of rat (based on maximal weight) was 0.615 c.mm. as compared with 0.305 c.mm.

Most interesting were the changes produced in the bones. In the unoperated controls, the lesions were those characteristic of a low calcium diet in the presence of an adequate amount of phosphate and conformed to the description of Pappenheimer, McCann and Zucker (23), and did not show the more extreme lesions described by Shipley, Park, McCollum and Simmonds (24) on their low calcium diet. The bones cut with less than normal resistance, but showed no marked deformity and little swelling at the epiphyses. Microscopically there

was a moderate uniform increase in the width of the zone of preparatory calcification and matrical deposition of calcium was somewhat defective. The trabeculae of the spongiosa were orderly and parallel in alignment, but rather thin, as was the cortex. Both trabeculae and cortex were surrounded by an osteoid border slightly in excess of the normal width. Osteogenesis was active, and there was no osteoclastic resorption or marrow fibrosis (Fig. 6).

The bones of the partially nephrectomized rats presented a striking contrast. In the gross, there was extreme rachitic deformity of the thorax with beading and angulation at the chondrocostal junctions, leading to narrowing of the thoracic cavity and extensive atelectasis of the lungs. The wrists and long bones showed typical epiphyseal swelling, and very characteristic rachitic cupping in the x-rays (Fig. 7a).

The histological picture differed in no respect from that of a florid low phosphorus rickets. There was great widening and irregularity of the cartilage, with almost complete failure of calcium deposition in the matrix. There was an extraordinary excess of calcium-free osteoid in the metaphyseal region and about the cortex (Figs. 8, 5).

Two representative experiments may be presented in tabular form. (Table III and Table IV.)

From these illustrative experiments several facts emerge. While the low calcium diet in itself brings about retardation of growth, the chronic renal insufficiency induced by experimental reduction of kidney tissue leads to more extreme stunting, in some instances (rat AE4) to dwarfism, comparable to that which accompanies renal rickets in human beings. In these cases, the skeletal lesions are extreme and the histological changes are those of florid rickets, with almost complete failure of calcium deposition in cartilage and osteoid.

While the low calcium diet in itself leads to a decided increase in the volume of the parathyroids, partial nephrectomy brings about a striking additional enlargement of the glands.

In the following experiment the low calcium diet was supplemented with 250 mg. per cent of CaCO_3 (Table V).

Rat AG4, operated upon at a very early age, died spontaneously after 29 days; it was greatly dwarfed, weighing only 36 gm. at death, as compared with 94 gm. in the litter mate control. The kidney was reduced to a thin walled hydronephrotic sac. Microscopically, the pelvis and tubules were greatly dilated, lined

TABLE III
Litter AD, Born Nov. 3, 1935, Low Calcium Diet

TABLE III

Litter AD, Born Nov. 3, 1935, Low Calcium Diet

No.	Operation	Total age days	Time after 2nd operation days	Final		PO ₄	Volume of parathyroid				Kidney lesions	X-ray	Bones		Rachitic lesions				
				Weight	Length		Ab- solute	Per 100 gm. (maxi- mum)	Per 100 gm. (final)				Femur	Tibia					
				gm.	mm.	mg. per cent	c.mm.	c.mm.	c.mm.				mm.	mm.					
1	Control																		
2	Nov. 13, 1935, partial L	79	—	122	—		0.649	0.532	0.532	—		±	25	31	+				
5	Dec. 4, complete R	98	75	115	15.3	10.23	1.418	1.050	1.262	+	+	+	28	36	+				
4	Dec. 4, complete L																		
	Nov. 13, partial L	98	—	140	16.0	7.86	0.844	0.562	0.603	—		+	26	31	+				
	Dec. 4, complete R	119	96	130	17.5	10.2	1.288	0.810	0.993	+	+	+	26	31	+				
3	Control																		
		119	—	232	19.3	7.86	0.492	0.262	0.262	—	±		30	35	+				

TABLE IV

Litter AE, Born Nov. 30, 1935

TABLE IV
Litter AE, Born Nov. 30, 1935, Low Calcium Diet

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TABLE IV

Litter AE, Born Nov. 30, 1935, Low Calcium Diet

No.	Operation	Total age	Time after 2nd operation	Final		Serum Ca	Volume of parathyroid				Kidney lesions	X-ray	Bones		Rachitic lesions				
				Weight	Length		Ab- solute	Per 100 gm. (maxi- mum)	Per 100 gm. (final)				Femur	Tibia					
		days	days	gm.	mm.	mg. per cent	c.mm.	c.mm.	c.mm.				mm.	mm.					
2	Dec. 9, 1935, partial R	69	59	76	14.8		0.530	0.696	0.696	+		+++	25	30	++				
4	Dec. 10, complete L	69	59	46.5	12.8		?	?	?	+		+++	19	25	++++				
5	Dec. 9, partial R	69	59	89	16.5	5.9	0.544	0.570	0.589	—		+	26	33	+++				
7	Dec. 19, complete L	69	—	105	17.5		0.362	0.331	0.342	—		+	27	33	+++				
8	Dec. 19, complete L	69	—	98	17.5		0.310	0.305	0.316	—		+	28	32	+				
	Control	69																	
Stock Diet																			
9	Control	69	—	124	20	10.6	0.236	0.190	0.190	—	—		33	37	—				
10	Control	69	—	130	19.5	10.6	0.161	0.124	0.124	—	—		33	38	—				

KIDNEY SUBSTANCE AND PARATHYROID GLANDS

TABLE V
Litter AG, Born Feb. 10, 1936, Low Calcium Diet + 250 Mg. Per Cent CaCO_3

No.	Operation	Total age		Time after operation	Final		Nonprotein nitrogen	Ca	Volume of parathyroid			Kidney lesions	Bones			Microscopic lesions
		days	days	days	Weight	Length	mg. per cent	mg. per cent	Absolute	Per 100 gm. (maximum)	Per 100 gu. (final)		X-ray	Femur	Tibia	
					gm.	mm.			c.mm.	c.mm.	c.mm.			mm.	mm.	
4	Feb. 15, 1936, partial L Feb. 19, complete R	38	29	36	11				0.3512	0.903	0.974	++	?	20	23	++
7	Control	39	91	94	218	15	65.4	9.92	0.118	0.1256	0.1256	—	?	24	31	—
5	Feb. 19, partial R Feb. 23, complete L	104	—	248	260	20	28.7	8.64	0.6587	0.3021	0.3021	++	—	33	37	++
8	Control	104	—	248	260	20	31.9	9.56	0.4889	0.1971	0.1971	—	—	35	40	—
9	Control	104	—	260	260	19.5	31.9	9.56	0.4304	0.1655	0.1655	—	—	35	40	—

with flattened cells. Casts were numerous. The glomeruli were small, the capsular spaces distended with albuminous precipitate, but there were no adhesions or hyaline changes. A few calcified casts were found in the collecting tubules. Quite marked changes were found in femur, tibia and ribs. The lesions may be described as combining the features of a mild rickets with those of osteitis fibrosa. The cartilage showed a few blunt imperfectly calcified prolongations towards the metaphysis, but in general hardly exceeded the normal width. The spongiosa was composed of irregularly disposed, thin trabeculae, separated by patches of fibrous marrow with numerous osteoclasts (Fig. 9). The cortex in the region of the metaphysis was thinned, and in places completely defective, being replaced by fibrous tissue with many giant cells. Further along the shaft, the marrow resumed its normal appearance, but the osteoid margin in some places was distinctly increased in width. The control rat, killed on the same day, had normal bones, the added CaCO_3 being sufficient to prevent obvious histologic lesions.

Other experiments in which lesser or greater amounts of CaCO_3 were added to the low calcium diet, have not given additional information.

Effect of Parathormone Injections upon the Bones of Partially Nephrectomized Rats

A possibility, which we have not seen discussed, is that the organism is rendered more sensitive to the effect of parathyroid hormone when there is a state of renal insufficiency. One of the factors leading in this direction may be the accompanying acidosis. Indeed, it has been well shown by Olsen (9) that the activity of paroidin, as measured by the urinary excretion of calcium after injection, is greatly enhanced when the animals are placed on an acid forming diet (NH_4Cl , meat). That the bone lesions resulting from repeated injections of parathormone in young rats are exaggerated, when the renal function is experimentally reduced, is indicated by the following experiment.

Litter AH, Mar. 20, 1936. Partial nephrectomy (R), at age of 8 days, Mar. 23 left kidney removed. Beginning Mar. 25 rats 2 and 4 of the litter and unoperated control 7 were given daily two intraperitoneal injections of parathyroid extract (Lilly), in all 60 to 90 units. The two operated rats died 7 days after the second operation, and the control was killed on the same day.

A histological study of ribs, tibia and femora showed distinctly more severe changes in the injected nephrectomized rats (cf. Fig. 10). Both in ribs and long bones, the submetaphyseal region is almost

replaced by fibrous tissue, containing only a few fragmented bone trabeculae. The cortex is reduced to a thin ribbon and entirely lost over considerable areas. Osteoclasts are extremely numerous in this region, but fibrosis of the marrow and osteoclasts are seen along the entire course of the rib and along the shafts of the tibia and fibula.

In contrast to these extreme lesions, the unoperated injected control shows only minor changes. The rib (Fig. 11) is normal save for a few osteoclasts and slight thinning of the cortex just below the cartilage. Tibia and femur show minimal lesions.

It would seem from this and other similar experiments that demineralization of the bones in young rats following parathyroid extract administration is intensified by the reduction of renal function. To determine whether this is caused by acidosis, alterations in calcium-phosphorus metabolism, diminished calcium intake or other unknown factors will require much further investigation.

DISCUSSION

It has been shown that the parathyroid glands react to a loss of functional renal tissue by an increase in volume, roughly proportional to the severity of the kidney damage. The immediate stimulus to this enlargement of the gland, which one may assume to signify increased functional activity, has not been determined. The suggestion of Schelling (25)⁴ and others that the enlargement in chronic nephritis is probably the "result of a functional demand on the glandules to rid the body of retained phosphates" is not wholly satisfactory. There is no direct evidence that the hyperphosphatemia *per se* is the chemical incitant to hyperplasia, and we are confronted with the fact that such hyperplasia occurs with equal constancy in low phosphorus rickets. Many of the reported cases of renal rickets have shown plasma phosphate values within the normal limits (Ellis and Evans, (26)). In the case reported by Elsom, Wood and Ravdin (27) of hyperparathyroidism with renal insufficiency, removal of an adenoma was followed by a permanent return of the serum calcium and PO_4 values to normal. In this patient, phosphate retention was never observed and could not have supplied the primary stimulus to parathyroid hyperplasia.

⁴ Schelling (25), page 278.

It seems idle to enter into a theoretical discussion of this point, since the precise mode of action of the parathyroid secretion is still obscure.

In reviewing the literature of renal rickets, there is a recurring suggestion that the hyperactive parathyroids play a part in the demineralization of the skeleton, bringing about the rachitic or osteofibrotic lesions which characterize the disease. Indeed, in certain cases occurring in adolescence or early adult life, in which there has been found a diffuse hypertrophy of all the parathyroids, the differentiation between primary and secondary hyperparathyroidism may be difficult or impossible.

Our experiments thus far have given no clear cut information as to the part played by the parathyroids in the production of the skeletal lesions. On a diet containing adequate calcium and phosphorus the parathyroid hyperplasia induced by partial nephrectomy was only exceptionally accompanied by slight osteofibrotic changes in the bones and these were never of an intensity comparable to those observed in "primary" hyperparathyroidism or in renal rickets.

On the other hand, when rats with experimental renal insufficiency were placed on a low calcium diet, the resulting skeletal lesions were greatly intensified. On a very low calcium intake, they took on the character of florid rickets indistinguishable from that produced by the usual low phosphorus rachitogenic diets. With a moderate addition of calcium, the picture was rather that of an osteitis fibrosa.

The crucial experiment, which has not yet been performed, will be to study the effect of experimental renal insufficiency in the absence of the parathyroids. In this way we should secure information as to the rôle played by these glands in the production of the skeletal lesions.

One of the purposes of this study was to reproduce, if possible, in rats the clinical picture of renal rickets or renal dwarfism in man. The outstanding features of this disease are stunted development, sometimes with delay or failure in the acquisition of the secondary sex characters; bone deformities, often genu valgum, with x-ray changes, sometimes suggesting osteitis fibrosa, sometimes florid rickets; chronic renal insufficiency, ending in marked nitrogen retention and uremia. Polydipsia and polyuria have been frequently noted. The blood calcium may be normal or slightly reduced, the inorganic phosphate, in the later stages, tends to be elevated.

The renal changes at autopsy are usually described as chronic interstitial nephritis, but recently attention has been drawn to the frequency of accompanying hydronephrosis and dilatation of the ureters, sometimes with vesical hypertrophy (Ellis and Evans (26), Roberts (28)). In some cases anatomic explanation for the renal obstruction has been found (phimosis, valve-like folds in the urethra (Ellis, (29))). In other cases (Ellis and Evans (26)) no organic stricture has been discovered, and a disturbance in sphincteric control has been assumed.

The histologic alterations in the bones have been studied in a number of instances. The descriptions vary from case to case, and there appears to be every gradation between a florid rickets, indistinguishable from ordinary rickets, to a picture identical with osteitis fibrosa in the adult (11, 24, 30-39). It would be impossible from the histologic changes alone to establish the diagnosis of renal rickets, as distinct from that due to vitamin D deficiency, on the one hand, or to primary hyperparathyroidism on the other.

The same variability has been found in the skeletal changes which follow experimental reduction of renal tissue in young rats. On a very low calcium intake they have resembled the lesions of florid rickets; with a moderate deficiency of calcium, changes are in the direction of osteitis fibrosa.

The stunting of growth and development in our rats is comparable to that noted in human cases of renal rickets. Our data on the blood calcium PO_4 and nonprotein nitrogen are not sufficiently numerous to report in detail, but so far as they go, agree with those reported in human cases of renal rickets, *i.e.*, hyperphosphatemia and high nonprotein nitrogen in the late stages of the experimental disease.

We believe therefore that we have induced a condition essentially like that which accompanies advanced chronic renal disease in childhood. This experimental renal rickets should be useful in working out the various unknown factors concerned in the human disease.

CONCLUSIONS

Reduction of renal tissue in young rats regularly leads to a marked increase in the volume of the parathyroid glands.

If partially nephrectomized rats are maintained on a low calcium diet, growth is stunted, and skeletal lesions are produced, of far greater

severity than can be ascribed to the dietary calcium deficiency alone. The picture closely resembles that found in cases of renal rickets in children.

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EXPLANATION OF PLATES

PLATE 51

FIG. 1. Rat AA2. Removal of right kidney. Cauterization of left. Survived second operation 37 days. Low power showing chronic nephritis, dilatation of tubules, casts, glomerular adhesions, interstitial fibrosis.

FIG. 2. High power, showing glomerular lesions.

FIG. 3. Rat B7. Rib. Area of marrow fibrosis with osteoclasts.

PLATE 52

FIG. 4. Litter AE. Low calcium diet. Rats 1 and 2, complete nephrectomy, right, partial left; rat 3, unilateral nephrectomy. Rats 4 and 5, unoperated controls. Rat 6, control on stock diet.

FIG. 5. Rat AE4. Partial nephrectomy. Low calcium diet. Extreme rachitic lesions with angulation.

PLATE 53

FIG. 6. Rat AC1. Unoperated control on low calcium diet. Slight increase in width of proliferating cartilage zone.

FIG. 7. Litter AC. Low calcium diet. X-ray of knee joint in operated (a) and control (b) rats.

FIG. 8. Rat AC1. Partial nephrectomy. Low calcium diet. Rib, showing very marked rachitic changes.

PLATE 54

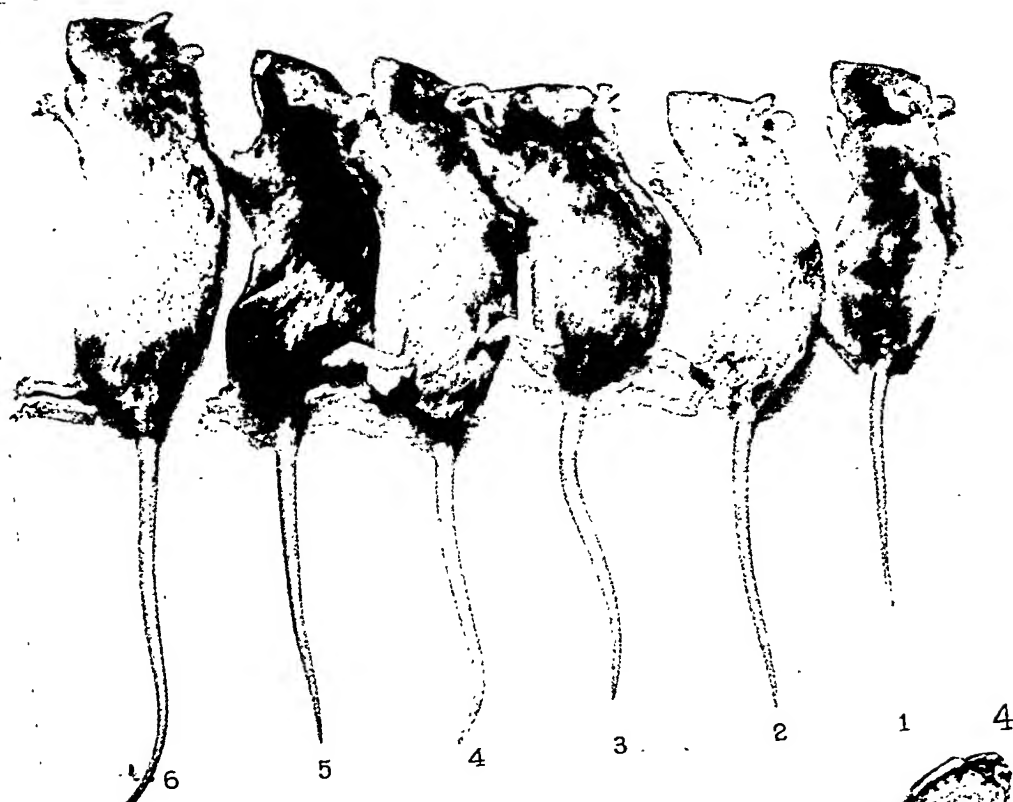
FIG. 9. Rat AG4. Partial nephrectomy. Low calcium diet + 250 mg. CaCO_3 . Tibia. Area showing fibrosis of marrow, rarefaction of trabeculae, many osteoclasts.

FIG. 10. Rat AH2. Low calcium diet + 150 mg. per cent added CaCO_3 . Partial nephrectomy. 80 units of parathyroid extract (Lilly) intraperitoneally in 4 day periods. Rib showing thinning and destruction of cortex and trabeculae, fibrosis of marrow and numerous osteoclasts.

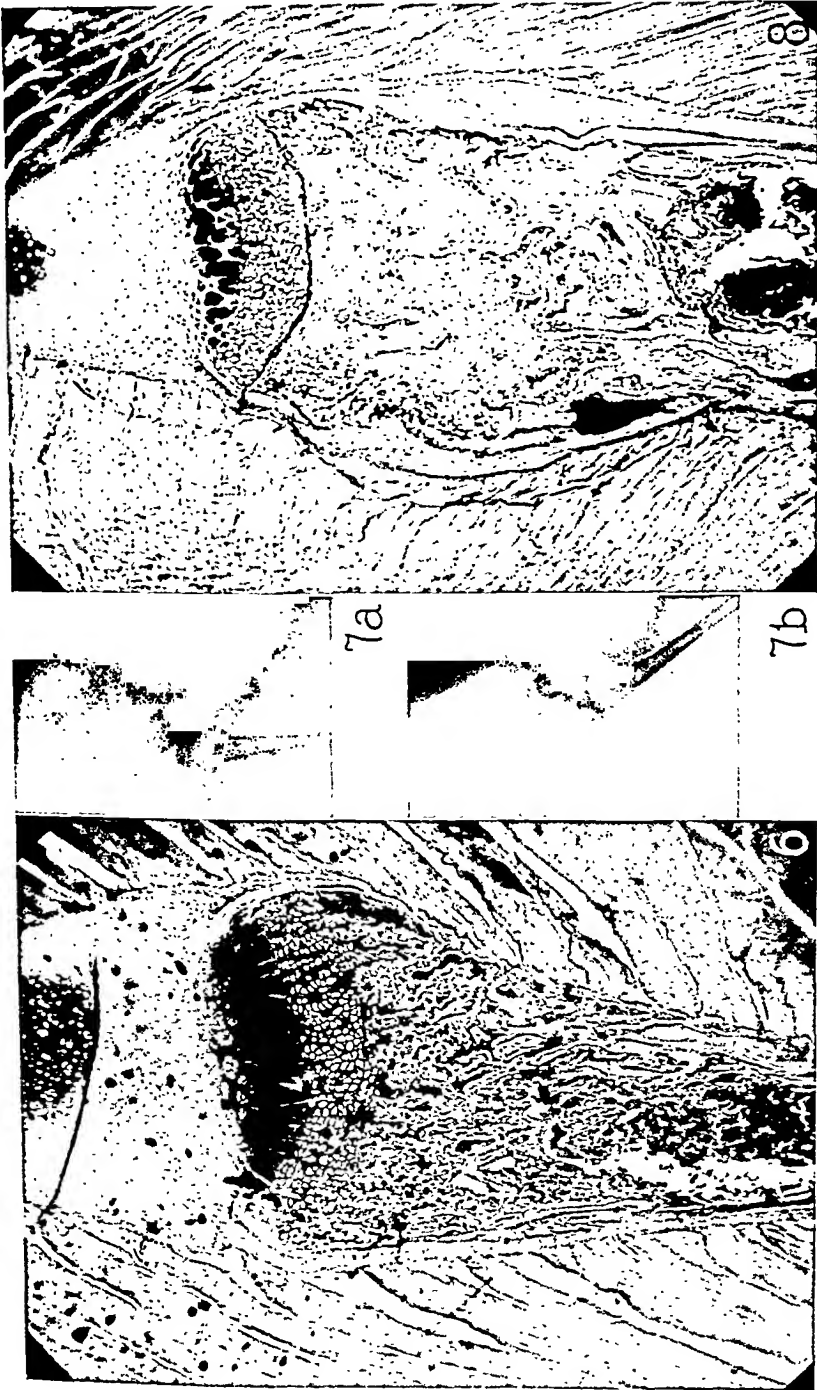
FIG. 11. Rat AH7. Unoperated control to rat AH2. Received similar amount of parathyroid extract. Practically normal rib.



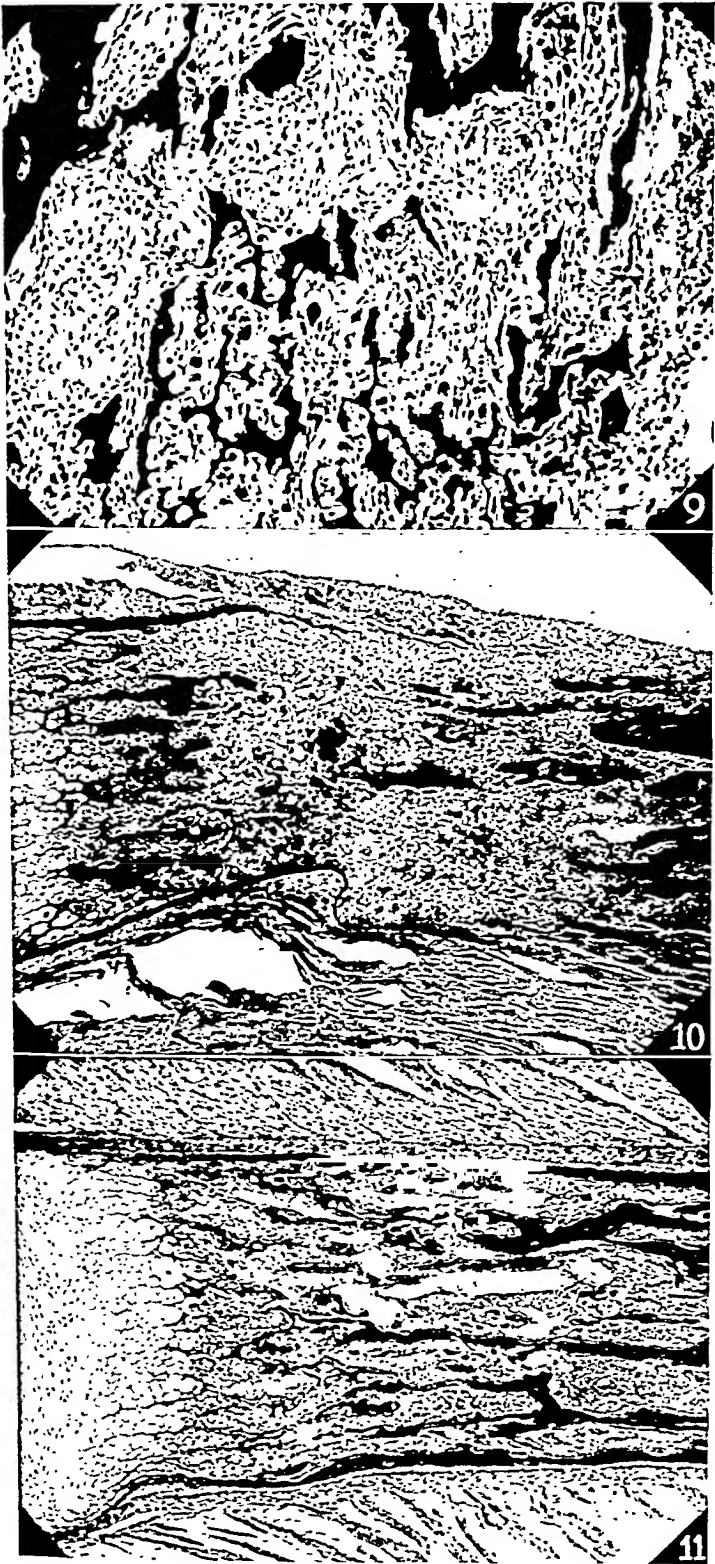
(Pappenheimer. Kidney substance and parathyroid glands)



(Pappenheimer: Kidney substance and parathyroid)



(Pappenheimer: Kidney substance and parathyroid glands)



(Pappenheimer: Kidney substance and parathyroid glands)

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